EFFECTS OF SUBLETHAL DOSES OF PYRETHROIDS ON MALARIA VECTORS

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Abstract

Pyrethroids are currently the most widespread class of insecticide in public health use, although their future may be limited by resistance. The present study investigates the effects of these compounds on mosquitoes, other than mortality immediately after exposure.

Longevity of both male and female *An. gambiae* was found to be significantly curtailed following sublethal exposure to the most commonly used pyrethroids, permethrin, deltamethrin and lambda-cyhalothrin. There was no direct effect observed on fecundity. Irritancy on direct contact with all 3 compounds was recorded, particularly with permethrin. Evening host-seeking flight activity was greatly reduced, and when females were treated with permethrin before release into a flight chamber, significantly fewer flew to a host to feed.

Experiments investigating *Plasmodium yoelii* and *P. falciparum* development in the Asian vector *An. stephensi* revealed that sublethal exposure to pyrethroids inhibited development of oocysts in the midgut. Similar experiments using organochlorine, organophosphate and carbamate insecticides found no effect.

Radio-labeled permethrin was traced inside the blood meal following exposure. However, none of the 3 pyrethroids were found to have a direct anti-malarial activity on cultured gametocytes. Likewise, permethrin did not affect exflagellation of *P. yoelii*. Time course experiments determined that for the reduction in oocysts there was a critical period of between 18 and 48 hours after the infective feed. Permethrin was not found to inhibit trypsin activity in assays, and the levels of trypsin in the midgut of blood fed treated and untreated females was not found to differ in a manner which could explain reduced infection rates.

It is clear that sublethal exposure of mosquitoes can lead to a wide range of potentially important effects in terms of the impact of pyrethroids in malaria vector control.

Acknowledgements

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CHAPTER 1. General Introduction

1.1. A brief history of insecticides

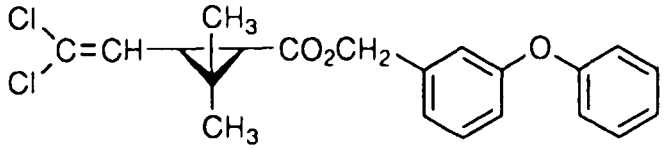
The first substances manufactured in any great quantity to kill insect pests in agriculture, public health or veterinary fields were naturally occurring minerals, such as lead and copper arsenates, fluorides and sulphur during the 19th century (Tessier, 1982). Yet some 2,000 years earlier, the Chinese were already utilising plant based compounds such as pyrethrum, derived from *Chrysanthemum* plants (Lhoste, 1964). In the West, it was not until the late 19th - early 20th century that the first specific, synthetic insecticides was produced, dinitro-orthocresol (1892), chloropicrin (1907), followed by the thiocyanates (Murphey & Peet, 1932) and phenothiazine (Campbell *et al.*, 1934) The discovery of a compound, dichloro-diphenyl-trichloro ethane (DDT), highly effective against a wide range of pest species by Müller (1939), bought great optimism that insect pests could be readily controlled. It was not long, however, before the widespread occurrence of resistant strains, and reports of excessively long persistence in the environment stimulated the search for more compounds. Within 10 years numerous other organochlorines were available for use, including dieldrin, hexachlorocyclohexane (HCH), aldrin, chlordane, heptachlor and endosulphan, amongst others (Slade, 1945). Unfortunately, like DDT before them, most soon lost favour due to emerging resistance and environmental concerns. During the 1950s, 1960s and 1970s, work on compounds containing phosphate or triphosphate groups yielded a range of important public health insecticides, the organophosphates, including malathion, dichlorovos, diazinion and pirimiphos-methyl. From the 1960s onward, these compounds were joined by another class, the carbamates, including carbaryl, propoxur and pirimicarb (Tessier, 1982).

1.2. Pyrethroid insecticides

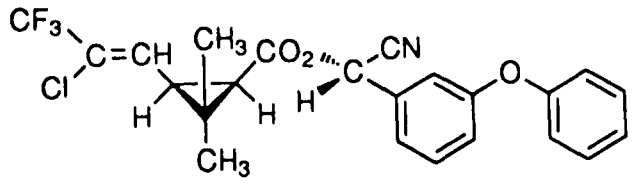
Despite the fact that it is likely that the Chinese were using pyrethrum extracts derived from the *Chrysanthemum* plants more than 2,000 years ago (Lhoste, 1964), it was not until the 1920s that any concerted research in the West was conducted in this area. Staudinger and Ruzicka (1924), conducted the first work on the specific insecticidal constituents found in the powdered dried flowers of the species *C. roseum* and *C. cinerariaefolium*, known as pyrethrum. The activity of these natural pyrethrins is due to 6 closely related esters of cyclopropane carboxylic acids, various isomers of pyrethrins, cinerins and jasmolins. Work was soon under way to produce synthetic compounds based on the natural pyrethrins in an attempt to increase potency and, more importantly, improve stability in daylight. Schechter *et al.* (1949) were the first to synthesis alletherin by esterification of allethrolone with chrysanthemic acid, followed by bioallethrin, a mixture of 2 sterioisomers (Schechter, 1949). When Gerdorff & Mitlin (1953) prepared a purified compound, later to be known as (*S*)-Bioallethrin, this became the first pyrethroid insecticide produced in large scale commercial processes. Having established that the structure of pyrethrum could be modified without affecting overall activity, a range of similar compounds, such as resmethrin and bioresmethrin, were synthesised during the 60's (Elliot *et al.*, 1967). Unfortunately, although these new chemicals had improved activity and lower mammalian toxicity, their stability in sunlight was still poor. In an attempt to overcome this serious deficiency, work was directed towards developing photostable pyrethroids, finally achieved by Elliot *et al.* (1973) who replaced the 2 vinyl methyl groups in resmethrin with chlorine, and α-benzylfuran with phenoxyphenyl. The result was permethrin, probably the best known and most widely used synthetic pyrethroid (see figure 1). Within 2 years of the discovery of permethin, several groups had modified the structure further, thus developing a range of compounds with the same photostability but greatly enhanced insecticidal potency. In Japan, Ohno *et al.* (1976) working with cyano groups substituted at the benzyl position developed fenvalerate and then cypermthrin, whilst Elliott *et al.*

(1974), found that dibromo groups in the ester acids led to higher insect toxicity compared with dichloro groups and produced deltamethrin (see figure 1). Similarly, a new compound developed by ICI Agrochemicals during the early 1980s replaced one of the chloro groups with a fluoro group to develop lambda-cyhalothrin (Tomlin, 1995). As a class, the photostable synthetic pyrethroids are currently the most important insecticidal weapon against the majority of insect pests. They exhibit very low mammalian toxicity, have medium term stability in the field (6 – 12 months), sufficient for most uses but without the problems associated with environmental contamination, and extremely fast action in low concentrations against most insect species. It is for these reasons that they currently play a leading role in public health programmes, and in particular against vector-borne human disease worldwide.

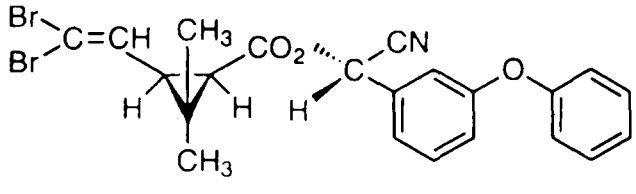
Figure 1. Chemical structure of pyrethroids investigated in this project.



Permethrin



Lambda-cyhalothrin



Deltamethrin

1.3. Pyrethroid impregnated bed nets

One of the most successful means of reducing malaria transmission through vector control, in recent years has been the increasing use of insecticide impregnated bed nets, a subject comprehensively reviewed by Curtis *et al.* (1990) and more recently by Lengeler (1998). Bed nets, or mosquito nets, have been a means of reducing night time biting by mosquitoes for many years. There are reports from Herodotus of fishermen sleeping with their fishing nets covering their beds to prevent "gnat" bites as early as the 5th century B.C. (Herodotus [translated by Godfrey], 1981). Bed nets themselves act merely as a physical barrier preventing mosquitoes gaining access to a sleeping individual, and have been in widespread use in some parts of the world for many years. As explained by Lindsay & Gibson (1988), the idea of treating such nets with insecticides is a relatively new idea and is designed to increase efficacy by overcoming some of the inherent problems of untreated nets such as: mosquitoes gaining entry through tears and holes; mosquitoes feeding on a limb in contact with the net; mosquitoes waiting in the room biting anyone who leaves the net during the night; and mosquitoes being diverted from those sleeping under nets to those who are not. The synthetic pyrethroids such as permethrin, deltamethrin and lambda-cyhalothrin, are ideal candidates for net impregnation. They are fast acting, have very low mammalian toxicity, are stable for 6 – 12 months on nets and relatively cheap due to the low doses used (Rozendaal, 1997). Impregnated bed nets have proven to be very effective under experimental conditions, and in many cases are readily accepted by most communities, and in some areas are preferred to residual house spraying (Lengeler *et al.*, 1996, Curtis *et al.*, 1998). The technique can be readily integrated into primary health care regimes, and nets can be made from local resources at minimal cost (Rozendaal, 1997). Aikins *et al.* (1998) studied the total financial implications of malaria control in The Gambian National Impregnated Bednet Programme and concluded they were cost effective and "one of the more efficient ways of reducing deaths in children under 10 years

in rural Gambia" A comparison of vector control in Tanzania conducted by Curtis *et al.* (1998) confirms the potential cost saving of impregnated nets over house spraying. This group used microencapsulated lambda-cyhalothrin, either on bed nets (4 villages), or sprayed onto inside surfaces of houses (4 villages). The level of control achieved compared to 4 control villages was very high, reducing the entomological inoculation rate by around 90 %, but was not different between the 2 treatments. However, the net impregnation used around one sixth the volume of insecticide as house spraying, and was more readily accepted by the villagers. The type of bed net fabric used and the actual insecticide or formulation chosen for impregnation vary from study to study, but the underlying principle remains the same. Several groups have investigated the most effective types of fabric for impregnation, with synthetics such as nylon or polyester often being preferred to cotton which tends to absorb excessive volume of insecticide (Mutuku *et al.*, 1992; Curtis *et al.*,1996) Other groups have compared the efficacy of various pyrethroids, preferential application doses or washable formulations on nets (Curtis *et al.*, 1992 & 1996; Njunwa *et al.*, 1991; Jawara *et al.*, 1988; Hodjati & Curtis, 1997; Miller *et al.*, 1995) Such work suggests the best compounds for nets are permethrin, deltamethrin and lambdacyhalothrin, with the latter two at lower doses, and that wash-fast formulations can prolong longevity.

Several groups around the world have reported encouraging results of impregnated bed nets against vector species. Magesa *et al.* (1991) showed impressive control of the major malaria vector, *Anopheles gambiae,* following introduction of nets to all inhabitants of some villages, as well as a fall in the entomological inoculation rates by more than 90%. In The Gambia, Lindsay *et al.* (1989a), found a similar degree of protection against the same species, with an estimated 90 % reduction in the number of bites per person in hamlets using permethrin treated nets. This was in contrast to work in the same area by some of the same group (Lindsay *et al.* 1989b), who concluded the use of untreated nets

would not be an effective means of malaria control as children were still likely to receive more than 1 infective bite per year. In China, both Li Zuzi in Guangdong Province, and Yang Jiuping in Sichuan Province, demonstrated a marked reduction of the vector species *An. anthropophagus* and *An. sinensis* indoors of between 93 % and 100 % using deltamethrin treatment of nets (Curtis *et al*, 1990). As well as demonstrating efficacy against the mosquito vector, encouraging results have more recently emerged regarding the clinical benefits of impregnated bed net use. In areas of low malaria endemicity, such as China, it has proven possible to reduce the overall incidence of malaria by as much as 60 % (Li Zuzi *et al.*, 1989). In The Gambia, Snow *et al.* (1988), Alonso *et al.* (1991) and D'Alessandro *et al.* (1995) have all reported evidence of reduced mortality and morbidity in children sleeping under impregnated nets. Similar reduction in childhood mortality and severe malaria morbidity was demonstrated on the Kenyan coast by Nevill *et al.* (1996). Even in areas of holoendemic malaria such as Northern Tanzania, Lyimo *et al.* (1991) have reported a reduction in slide positivity and fever episodes after the introduction of permethrin impregnated nets. Interestingly, this study also found there were no corresponding clinical improvement in villages using another form of vector control, DDT spraying. When impregnated bed nets are used in combination with anti-malaria drugs it is much easier to see a clear clinical benefit of their use, as residual infections are cleared and only new infections are recorded (Msuya & Curtis, 1991). In controlled trials with lambda-cyhalothrin impregnated nets and / or the drug combination dapsone-pyrimethamine (malarone), conducted by Marbiah *et al.* (1998) in Sierra Leone, both treatments in isolation gave a similar reduction in spleen rates and clinical episodes in children (nets 49 %, drug 43 %). When used in combination, this level of protection rose to 72 %. Similarly, in Tanzania, Msuya & Curtis (1991) found that once cleared by antimalarial drug use, parasitaemia remained low far longer in villages using treated nets. Similar studies conducted by Maxwell *et al.* (1999), again in Northern Tanzania, confirmed both Lambda-cyhalothrin and alphacypermethrin treated nets were highly

effective at preventing reinfection with malaria once existing infection had been cleared with chlorproguanil-dapsone.

One common criticism of impregnated bed nets is the fact that in many areas, the malaria vector species begin feeding shortly after dusk and for some hours before the adult population retire to their beds. Indeed, such a confounding factor was suggested by Graves *et al.* (1987) as to why malaria incidence was reduced in Papua New Guinea in under 5's who went to bed under treated nets early, but not in older children who stayed up later. In an attempt to overcome this, several workers have been investigating the possibility of pyrethroid impregnated eaves nets and window curtains. Lindsay & Snow (1988) highlight the problem of mosquito entry via the gaps between roof and wall in the majority of rural dwellings. Lindsay *et al.* (1995) subsequently went on to prove houses with open eaves were one of several factors associated with increased risk of malaria among Gambian children. In Burkina Faso, Majori *et al.* (1987 & 1989) recorded a dramatic reduction in indoor spray catches of both the Phlebotomine sandfly vector of *Leishmania*, and the local malaria vectors *An. funestus* and *An. gambiae*, following introduction of cotton netting door and window curtains impregnated with permethrin. In the same country, Habluetzel *et al.* (1997), found a significant reduction in infant mortality and parasitaemia following a 2 year controlled study using 1000 mg/m^2 permethrin on window curtains, demonstrating how effective such measures can be if implemented on a large scale. From experimental hut experiments reported from Tanzania by Lines *et al* (1987), it was discovered that using permethrin impregnated netting around the eaves alone did not provide significant protection to those sleeping within. However, treated curtains did provide moderate protection but not as effective as bed net use. One very interesting observation was provided by this study which had 2 children sleeping in the huts, one under an impregnated net, the other in the open. It was found that the number of bites received by the uncovered child was significantly lower than if neither

child had a treated net, in contrast to what happens if the bed net had been untreated. This supports the view that impregnated nets can provide a community wide benefit even when not all individuals are using them, presumably due to the high mosquito mortality or repellency effect of the insecticide as reported by Hossain & Curtis (1989). It is also interesting to note that use of impregnated nets can confer additional benefits to the user, such as the control of bed bugs, *Cimex hemipterus*, headlice, *Pediculus capitis*, (Lindsay *et al.*, 1989c), and possibly scabies and domestic insect pests such as cockroaches and houseflies too.

1.4. Sublethal doses of insecticide

The term refers to those effects observed upon an organism which has survived the characteristic mortality immediately or shortly following contact with a toxic substance. There are numerous possible causes of incomplete mortality. In this work for example, using an insecticide on mosquitoes, the most commonly encountered reasons for failure to achieve total kill include :

1.4.1. Insufficient contact time with the insecticide

a) Behavioural resistance, whereby the mosquitoes avoid contact with the treated surfaces, for example, when normally endophilic species become exophilic, thereby evading contact with insecticide sprayed on the inner house walls (Suwonkerd *et al.*, 1990 and Li *et al.*, 1983).

b) Shortened contact with insecticide, due to the irritation caused to the insect during contact with substances exciting the peripheral nervous system (Sharp *et al.*, 1990 and Chareonviriyaphap *et al.*, 1997).

1.4.2. Inadequate dose of insecticide available

a) As a result of incorrect application procedure.

b) Reduction in activity due to decay of original compound either by age or environmental factors, including light, temperature and moisture.

1.4.3. Insect resistance mechanisms

a) Biochemical methods of metabolism or detoxification of the insecticide. A range of enzymes have been detected which provide individuals with resistance to specific or whole groups of insecticides. Commonly encountered enzymes which confer insecticide resistance in this manner, such as glutathione-s-transferase, esterases and monooxygenase P450, have been reviewed by Corbett *et al.*, (1984) and more recently, Hemingway and Ranson (2000).

b) Alteration of the target site. When the insecticide acts on a specific tissue or enzyme within the mosquito it has been found that slight modifications to them can result in reduced mortality. For example, Hemingway and Davidson (1983) reported that in resistant *An. atroparvus* an altered acetylcholinesterase was not affected by organophosphate or carbamate insecticides. The presence of this slightly modified enzyme results in resistance of a number of insect species despite the fact that active, non-metabolised insecticide is present within the body. As one particular target site may be the focus for a number of different insecticide groups this type of mutation leads to cross-resistance, a particular problem for field use in vector control programmes, as indicated by Herath and Davidson (1981) in Asian *An. culicifacies*, and several other important species worldwide (Hemingway and Ranson, 2000).

c) Reduced penetration of the substance. Soderlund & Bloomquist (1990) detail work which indicates thickening of the cuticle may be responsible for preventing lethal doses of substances entering the body.

1.5. Sublethal doses of pyrethroids

Like the other classes of insecticides, the pyrethroids may be encountered by mosquitoes at sub-optimal levels. The application of pyrethroids can be at a dose insufficient to give total mortality for the generally short period of exposure. This may come about due to the perception of relatively high costs of these compounds leading to an overzealous economising, by managers of local spray teams, for example. A review by Curtis (1994) evaluating the evidence for continued use of compounds such as DDT to control malaria vectors concludes that, in fact, use of DDT is only slightly cheaper than pyrethroids. This is due mainly to the vastly reduced application rates required by the latter group, and appreciation of this ought to encourage applications of pyrethroids at optimal doses, thus reducing selection pressure for resistance by use of low doses which allow survival of resistance heterozygotes.

A working paper by Georghiou (1975), reviews observations from many parts of the world, and provides evidence of insecticide resistance in vector populations following use of similar compounds on crops in the same areas. Such effects are implicated in a number of ways ;

i) The appearance of vector resistance prior to the use of compounds for mosquito control.

ii) Higher levels of vector resistance in areas of active agricultural spraying.

iii) A correlation between resistance levels in vectors and the amount of agricultural pesticide use.

iv) Seasonal variation in vector resistance corresponding to seasonal crop spraying.

v) Similarities in spectrum of resistance between public health and agricultural pests.

vi) Suppression of vector populations during agricultural spraying.

As pyrethroids are now widely used as agricultural insecticides, this too may increase the likelihood of the development of pyrethroid resistance. Such an effect may be particularly apparent where there is contact of larvae with low level residues from agricultural pesticide use, the spraying of rice fields, for example. Such concerns have been raised by Lines (1988).

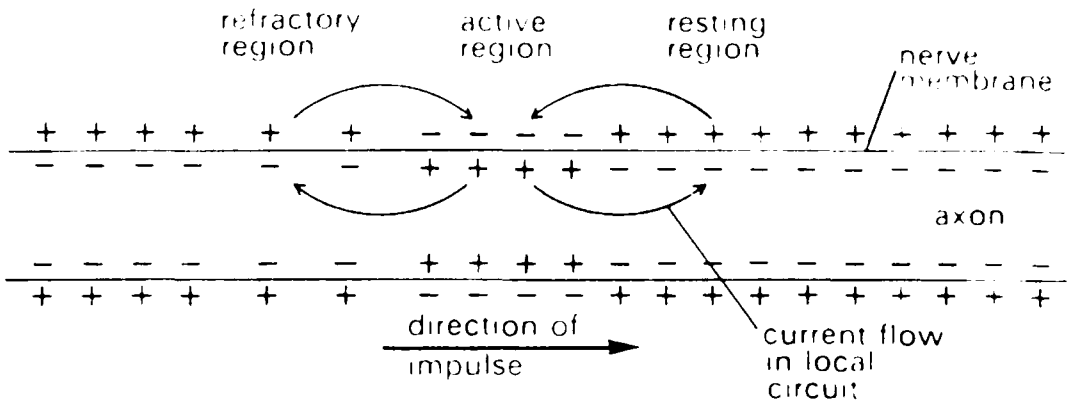
Laboratory experiments placing mosquitoes in contact with pyrethroids have clearly shown that individuals are quickly irritated, tending to avoid prolonged resting on, or movement over, treated surfaces (Miller & Gibson, 1994). When pyrethroids are impregnated onto bed nets in the field similar observations have been reported. Jinjiang *et al.* (1988) in Guangxi Province, China, concluded that one of the beneficial effects of permethrin used on nets was an effectively repellent action against the malaria vectors. Similar effects of mosquitoes exiting houses treated with pyrethroids are often reported, whether in the case of residual sprays (Taylor *et al.*, 1981), impregnated bed nets (Lines *et al.*, 1987) or pyrethrum coil smoke (Hudson & Esozed, 1971). Both in the laboratory and in the field this phenomenon of reduced contact time due to irritancy would lead to a sublethal dose being received by an individual which manages to survive the initial exposure. It is not clear what causes deterrency (insects not entering houses) of impregnated nets used in the field, the low vapour pressure of pyrethroids makes it unlikely there is a true airborne repellent effect (Tomlin, 1977), and some suggest it is due

to the solvents used (Lindsay *et al.*, 1991), or pesticide residues on airborne dust (Smith & Webley, 1969).

1.6. Insect nerve impulse

In general, the conduction of nerve impulses within insects can be considered similar to that in the mammalian nervous system, with a membrane potential existing between the inside and the outside of the axon. The magnitude of this potential varies but Chapman (1969) regards the general figure as being around -70 mV. As in other animal axons, this resting potential is brought about by the active removal of Na^+ ions from the inside of the membrane to the outside, resulting in a proportionally higher concentration of Na^+ ions outside, followed by a flow of K^+ ions into the axon. The movement of these ions results in an equilibrium being reached with the inside of the axon negatively charged with respect to the external surface and surroundings.

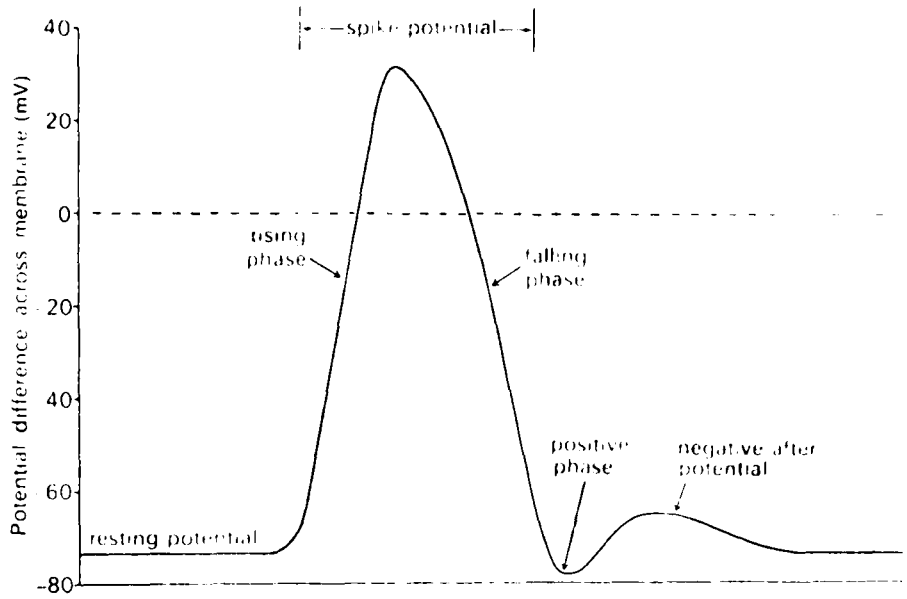
Figure 2. Diagram of impulse conduction along an insect nerve axon. (Chapman, 1969)



An impulse along the nerve takes the form of a disruption in this resting membrane potential, which is known as an action potential. When a depolarisation of the axon

occurs, the membrane experiences a sudden change in its permeability to Na^+ ions, and these rush into the axon causing a rapid rise in positive charge. After this action potential has occurred the resting equilibrium is rapidly restored as the membrane becomes more permeable to K^+ ions and the Na^+ ions are once again actively pumped out. Once initiated, an action potential passes along the axon with the changes in potential becoming self-regenerative. Figure 3 shows an idealised action potential, as described by Chapman (1969), and two additional stages of the impulse can be identified as follows. After the falling phase it is seen that the level falls just beyond the resting potential, caused by a delay in the suppression of increased K^+ permeability.

Figure 3. **Diagram of changes in potential difference across the plasma of an axon during the passage of an impulse (Chapman, 1969)**



This slight drop in potential, known as the positive phase, is reversed very rapidly by the fact that during the action potential there was a build up of K^+ ions on the axon outer membrane, and this results in a net influx of these ions. This final passage of K^+ ions raises the potential slightly higher than the resting potential, known as the negative after

potential, but soon the local concentration of these ions dissipate and the membrane potential returns once more to its resting value. There is a critical membrane potential difference known as the threshold potential which, when reached, will always initiate an action potential and cause an impulse along the nerve. In insect axons this figure is quoted by Corbett *et al.* (1984), to be around 10 - 20 mV more positive than the normal resting potential. If, for example, the negative after potential is increased above this figure, then repetitive firing in the membrane would result, rather than re-establishment of the normal nerve equilibrium.

1.7. Mode of action of pyrethroids

From the earliest studies it was clear that pyrethroids interfered with the insects normal nervous system activity. Pyrethrins, the precursors of modern synthetic pyrethroids were first demonstrated as being direct stimulants to insect nervous systems by Lowenstein (1942). By the early sixties, Narahashi (1962), had reported that in the giant axons of the cockroach, *Periplaneta americana,* the pyrethroid allethrin caused an increased negative after-potential which went on to eventually block further nerve transmission. This early work also showed that, at lower doses, allethrin still increased the negative after-potential but did not result in the blocking of subsequent impulses. The importance of this effect of pyrethroids on the negative after-potential in the insect nerve is apparent when viewed in association with the physiological basis of impulse transmission described earlier.

1.8. Repetitive nerve impulses

Work by Welsh and Gordon (1947) and Yamasaki and Ishii (1952) on isolated nerve fibres showed repetitive discharges from a single stimulus when exposed to natural pyrethrins. Following application of most pyrethroid insecticides to isolated insect nerve preparations, the most frequently reported symptom is that of repetitive discharges resulting from a single stimulus. The early work using the then newly developed synthetic pyrethroids

was carried out by Narahashi (1962) on *P. americana* giant axons. This has now been repeated by numerous other workers, in a range of insect species, and on a variety of nervous tissues. Adams and Miller (1979) reported repetitive firing of motor neurons in the house fly, *Musca domestica*, while Clements and May (1977) observed it in the peripheral nervous system of *Schistocerca gregaria*, the desert locust. It was the work of Narahashi in the early sixties that first postulated that the repetitive discharges caused by pyrethroids are due to a increased negative after-potential (see figure 3). The result of this increase was that the threshold potential was surpassed and a second action potential initiated, and possibly many more subsequently. Depending on the dose of pyrethroid used, the repetitive discharge of the nerve may also be accompanied by a subsequent desensitisation, where no further impulses can take place at all. The higher the dose the more is the likelihood of this total nerve block occurring.

1.9. Effects on the Sodium channels

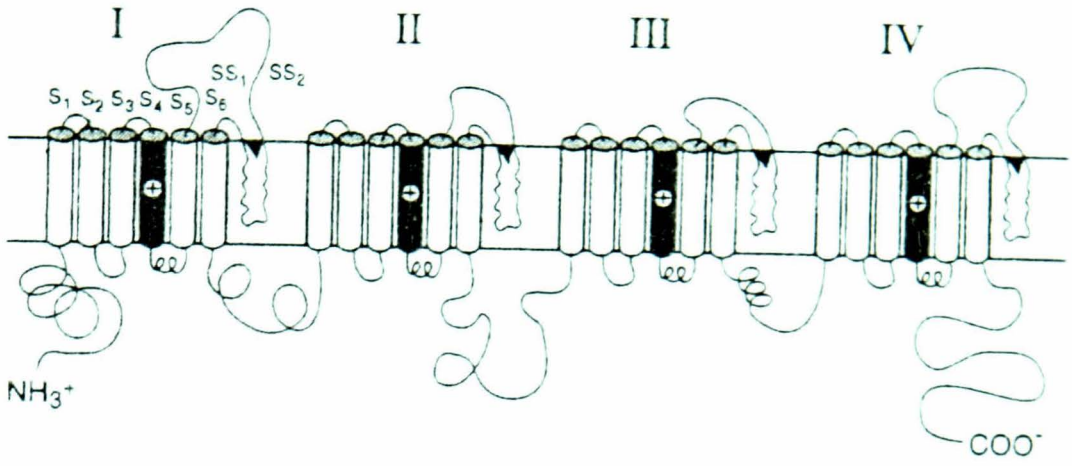
Lund and Narahashi (1981) using nerve isolates from the squid, and Vijvernberg (1982) working on frogs, proposed a plausible molecular mode of action to explain this phenomenon of increased negative after-potential. It is now believed that pyrethroids act on the sodium channels within the membrane of the nerve, and cause some of these to delay their normal closure following depolarisation. When the voltage-clamp technique was first developed by Narahashi and Anderson (1967) for use on giant squid axons poisoned with allethrin, it was discovered that both sodium and potassium channels were affected. The reason why this technique was so useful was that it allowed the nerves under test to be perfused internally, as well as externally, with buffers of known ionic constitution. In this way any individual ionic current component can easily be eliminated by its omission from the perfusate. For example, by removal of the potassium ions, and by blocking the K^+ channel with cesium ions it was possible to study the precise movement of sodium through the Na^+ channels only. Narahashi (1986) looked back on his earlier work and identified

the voltage-clamp technique as "A quantum jump in our understanding of the mechanism of action of pyrethrum insecticides." Using this technique Narahashi and Anderson (1967) concluded that the peak amplitude of sodium current was suppressed, the falling phase of the sodium current was prolonged, and that the steady-state potassium current was reduced. They also stated that the most sensitive of these changes caused by allethrin was the increased falling phase when the sodium current is prolonged, and they concluded that this was the major effect of pyrethroid poisoning. Wang *et al.* (1972), agreed with these findings. If Narahashi considered use of voltage-clamp techniques to be a "quantum jump" in the 1960s then the development by Neher and Saakmann (1976) of the patch clamp technique must rate as another. This method permitted the direct recording of currents passing through individual ion channels, and was subsequently modified by Hamill *et al.* (1981) to provide clear indication of Na^+ ion currents through a single sodium channel. In principle, a small patch of membrane is isolated by placing an adapted glass pipette tip over an area 3 - 5 μm in diameter. The pipette is filled with standard Ringer's solution and connected to ground by an electrode which also enables the measurement of currents through the circuit. Horn and Patlak (1980) adapted this technique to isolate patches with the internal axon membrane facing outside (inside-out patch), as opposed to the normal situation (outside-out patches). Yamamoto *et al.* (1983) used patch clamps on tissue cultured neuroblastoma cells (N1E-115 line), and recorded the action of tetramethrin on sodium currents in single channels. Results showed that, following exposure to the pyrethroid, the opening times of the channels fell into two distinct groups. Some sodium channels were at a constant open period of around 1.8 ms, while others were held open for an average of 16.6 ms. It appears that there are two populations of sodium channels in the membrane, and only one of these is bound by tetramethrin. As effects are clearly visible in the tissues even when only part of the total complement of sodium channels are affected, this may explain why pyrethroids often show such dramatic symptoms and high mortality at relatively low doses.

1.10. Site of action on the sodium channel

Some molecules have clearly defined sites of action on sodium channels. Batrachotoxin (BTX) has a well documented specific binding site within the sodium channel, known as the 'inactivation gate receptor' (Khodorov, 1978; Dubois and Khodorov, 1982). By binding to this site inside the sodium channel, Tanguy *et al.* (1984) found that it prevents channel closure during depolarisation, thus increasing the sodium current, and therefore resulting in similar effects to that described for pyrethroid poisoning. However, when BTX is used to modulate sodium channels, no significant change in the sodium tail current is seen, unlike that reported after pyrethroid treatments by Narahashi (1986), so this does not appear to be the same site of binding. When BTX is bound to the sodium channels and then tetramethrin is also added, the expected increase in sodium current was observed during a depolarisation (Narahashi, 1986). When this was repeated with internal application of tetramethrin to the axon, the sodium current was extended once again, but this time the characteristic extension of the tail current was also recorded. The pyrethroid was clearly exerting its own effect even though the inactivation gate receptor site was already occupied by the BTX. As this indicates that pyrethroids do not bind within the channel, the most likely site for them to do so is the lipid phase in the immediate vicinity of the sodium channel; this is quite likely because of their high lipid solubility. To test this hypothesis further, and to eliminate the possibility that pyrethroids do bind within the sodium channel but at a different site to that of BTX, it is helpful to look a little more closely at the channel itself (see figure 4). The sodium channel should not be considered merely as a tube across the membrane, as it acts selectively as a passageway for a variety of ions and cations, any of which may bind or interact with the channel during their journey.

Figure 4. **Diagrammatic representation of an insect sodium channel.** Subunits I and IV are in close proximity within the axon membrane which results in the characteristic cylindrical ion pore arrangement (from Zlotkin, 1999)



If the pyrethroid is truly binding near to, but outside, the channel then it is to be expected that the normal passage of cations would be unaltered while the channel remained open. Yamamoto *et al.* (1986) studied the temporary binding of Ca^{++} within the sodium channel and found this to be unchanged after treatment with tetramethrin on squid giant axons. This *in vivo* work further corroborated the *in vitro* studies made earlier with cultured neuroblastoma cells (Yamamoto *et al.*, 1984). As tetramethrin was clearly not interfering with cation selectivity within the channel whilst it was open, it seems unlikely the insecticide was binding inside. This leaves the hypothesis of lipid phase binding near to the channel, from where they modify the gating kinetics, as the most logical one for the site of pyrethroid action.

1.11. Effects on calcium channels

Calcium is known to play a critical role in a variety of nerve and muscle functions, so any effect on transport or its levels within the membrane may be an obvious target for insecticide action. Studies were undertaken using the patch clamp technique on cultured neuroblastoma cells, in which two types of calcium channels were discovered (Tsunoo *et*

al., 1985). These two types of channel were found by Yoshii *et al.* (1985) to exhibit different physiological and pharmacological properties and both were later studied by Narahashi (1986) in the presence of tetramethrin. In type I calcium channels tetramethrin was found to reduce the peak current by 70 - 80 %, whereas it only reduced that of type II by around 20 - 30 %. The significance of the large effect on type I channels is uncertain, as their specific function is not known. Type II channels are believed to be related to the controlled release of neurotransmitter from nerve terminals (Narahashi, 1986), but as the effect of tetramethrin on this type is much smaller, Narahashi concluded that overall effects of pyrethroids are less significant on the calcium channels than on the sodium channels. Orchard and Osborne (1979) reported that a number of pyrethroids noticeably increased electrical activity in isolated neurosecretory neurons in the stick insect, which are known to be either wholly or partly activated by calcium ions. This, as well as further work by Orchard (1980), suggests that although these effects may not, on their own, be life threatening to the insect, alteration in the calcium currents may result in some physiological changes detrimental to the adult in its later life. As less work has been carried out on the calcium channels than the sodium channels it is less clear in this case exactly how the pyrethroids interact. Corbett *et al.* (1984) conclude that the probable mode of action is on the natural pathway of activation, or by directly stimulating the membrane to increase its resting spontaneous activity.

1.12. Effects on non-nervous tissues

Many pyrethroids have been shown to elicit direct and sustained muscle contraction, without any apparent associated nervous activity. Clements and May (1977) blocked motor axons associated with skeletal muscles in the locust and recorded full contractions after application of low pyrethroid doses. They concluded that the substances were acting directly upon the muscle rather than on the motor neurons or neuromuscular synapses.

1.13. Effects on neurosecretory cells and hormonal control

Because pyrethroids act on neurosecretory cells and hormone control these insecticides have detrimental effects throughout the insect body. In a review of effects of insecticides on neurosecretory cells, Osborne (1986) presents many examples of depletion of neurosecretory granules in many tissues following exposure. Osborne (1980) reports abnormal release of products from the neurohaemal organs in stick insects following contact with permethrin. Norman (1980) found treatment of *Rhodnius prolixus* with bioresmethrin induced loss of neuroglandular cell granules from the corpora cardiaca. The same author also demonstrated excessive release of hyperglycaemic hormone in blowflies treated with bioresmethrin or permethrin.

1.14. Summary of pyrethroid poisoning

Clearly the major factor leading to poisoning of insects by pyrethroids is the activation and prolonged repetitive discharges within the central and sensory nervous system. The resultant symptoms of hyperexcitation, ataxia, tremors and convulsions are explained by this mode of action. In addition, there is abnormal release of hormones into the body as a consequence of stimulation of neurosecretory cells as described by Maddrell (1980) and Osborne (1986). Corbett *et al.* (1984) also suggests that the degeneration of vital organs may result from water loss from the insect integument following pyrethroid contact, which Gerolt (1976) describes as a common phenomenon observed with other insecticides acting upon the nervous system such as the carbamates and organophosphates. Exposure to pyrethroids can also result in more readily observable effects on the nervous system. Osborne (1986) found that even low doses of deltamethrin resulted in ultrastructural damage to the nerve fibres themselves, showing up as electron opaque areas under the electron microscope. Given the relatively poor regenerative capability of nervous tissue in general, it is understandable that, even if an insect survives exposure, the damage

caused will severely debilitate the individual. An overview of the neurotoxic effects of pyrethroids is presented by Soderlund and Bloomquist (1989).

1.15. Effects of sublethal insecticide poisoning

In view of all the effects described above it is perhaps not surprising that one observes a wide range of major effects bought about by contact with insecticides at levels which do not induce immediate mortality. The majority of the substances in use as pesticides today act by interfering with the normal processes within the insects nervous system (Corbett *et al.*, 1984). Clearly, any changes in this vital system will result in widespread effects on all physiological and behavioural phenomena which are directly or indirectly controlled by it. With so many bodily and behavioural functions linked to the CNS or peripheral nervous system, an individual surviving insecticide treatment will still be liable to encounter further consequences due to prolonged damage to its system or by low levels of the active substances remaining within the integument. When such effects prove to be detrimental to the insect's survival, fecundity, behaviour or other factors, such as its ability to transmit disease, these sublethal effects should be regarded as complementary to the primary purpose for pesticide application, that of immediate mortality. There are two main reviews on sublethal doses, the first was Moriarty (1969) which surveys a range of early synthetic insecticides, and Haynes (1988) which concentrates mainly on the effects upon insect behaviour.

1.16. Aims of the work described in this thesis

The introductory review has considered the development, public health use and mode of action of insecticides, and in particular the pyrethroids. It has also introduced the causes and effects of sublethal contact with such compounds in many insecticide classes and insect species. This thesis investigates a range of effects on the biology, physiology, behaviour and parasite susceptibility of sublethal exposure of anophelines to three widely used

pyrethroids, permethrin, lambda-cyhalothrin and deltamethrin. Each chapter considers a specific topic, or closely-related topics, of relevance to the ability of the insect to transmit malaria, such as adult longevity, feeding behaviour and oocyst development. The overall objective of the study was to determine the range and magnitude of effects of sublethal pyrethroid contact on vector mosquitoes in relation to disease transmission and likely impact on vector control measures.

CHAPTER 2 General techniques

2.1. Aims of this chapter

This chapter outlines the more general procedures which were used throughout the study and which are therefore common to many of the subsequent chapters. Full details of all mosquito species and the various strains used throughout the study are provided and commonly used techniques, such as standardised insect rearing and insecticide exposure, are presented.

2.1.1. The importance of standardised insect rearing

In any biological experiment particular attention must be paid to selection of the test subjects. To enable accurate conclusions to be drawn it is necessary to be confident that every procedure, and each replicate within it, have as near identical characteristics as possible. When using living organisms this is particularly pertinent as natural genetic variation within the individuals can be compounded by environmental variation. The mosquitoes used in each of the experiments that follow have been in permanent culture at The London School of Hygiene & Tropical Medicine (LSHTM) for a number of years as closed colonies of a few hundred individuals, occasionally passing through accidental "bottle necks" of as few as 10 individuals. As a consequence the mosquitoes can be considered to be relatively genetically uniform. All adults used have been reared in the same fashion from single egg batches and then maintained under strict environmental conditions kept constant throughout the experimental period. Such procedures should eliminate the variation in longevity, fecundity and body size which was described by Hodjat (1969) when *Dysdercus fasciatus* were reared under unfavourable conditions. Reduced longevity, egg numbers and an increase in normal activity were also observed in the same cotton pest species by Hodjat (1971) when individuals were overcrowded or offered an inferior diet.

Variation in adult mosquitoes is important too, as Nayar & Pierce (1980) demonstrated that by reducing the concentration of sugar in the diet there were significant changes in longevity and oviposition rates. Gomulski (1988) also reported that larval nutrition and rearing densities influenced physiology of the emerging adults when observing wing length and body size in anopheline mosquitoes. Such a variation in body size has been shown to influence blood meal volume in *An. stephensi* by Ichimori (1989), emphasizing that standardisation of rearing techniques is vital when one is investigating egg production or infection rates. Takken *et al.* (1998) demonstrated that not only did larger *An. gambiae* females contain significantly greater reserves of protein, glycogen and lipid than their smaller counterparts, but they also found increased longevity and a stronger host response in larger individuals. Experiments by Baqar *et al.* (1980) demonstrated that infection of *Culex tritaeniorhynchus* with West Nile virus was much higher in adults which had been reared as larvae under sub-optimal nutritional conditions. Roberts (1998) designed a series of experiments which suggest that at least some of these effects are a result of chemical factors as well as physical or nutritional factors. Larvae of *Cx. sitiens* were reared at a range of densities with the highest, not surprisingly, resulting in delayed pupation. However, when low densities of larvae were subsequently reared in the same water from the high density experiments these too had significantly extended larval periods. This chemical retardant was found to be unstable, as subsequent batches of larvae using the same water were unaffected. Perhaps even more surprising was the fact that the effects of early exposure to these retardant chemicals was irreversible, as delayed pupation was not corrected by moving young larvae to a less crowded, clean water.

Takahashi (1976) found that the infection levels of *Cx. tritaeniorhynchus* fed Japanese Encephalitis virus varied greatly under differing insect rearing temperatures, and to some extent depending upon level of larval nutrition. The author believes that virus

development was inhibited as a result of "relatively slow physiological activity of mosquito tissues" at lower temperatures, rather than a direct effect of temperature on the virus itself. A similar effect on *Plasmodium* development in mosquitoes is likely to be found as parasite growth and maturation in the invertebrate host is influenced greatly by temperature (Bruce-Chwatt, 1980). The clearest demonstration that standardised rearing conditions are vital for laboratory vector / parasite studies is the work by Kitthawee *et al.* (1990) with *An. dirus*. They found a significant difference between large and small mosquitoes, not only in survivorship where larger individuals lived longer, but also in numbers of oocysts which developed following infection with *P. falciparum*, probably as a result of larger females imbibing a greater volume of blood leading to increased infection levels.

2.1.2. Insecticide treatment

Ever since the advent of insecticides against insect vectors it was clear that there was a need for an easy, fast and reproducible method for testing efficacy and resistance status throughout the world. To this end, the World Health Organization (WHO) introduced a standard methodology based on impregnated papers lining a simple connecting tube apparatus, as detailed fully in WHO (1963). This technique is still used for testing new compounds and monitoring efficacy of existing compounds on field populations worldwide. Having this one established method should mean that results from different groups are consistent and reproducible.

In some cases the standard WHO paper lined tube method, can be criticised as being rather artificial. A clear example of this is when considering application of pyrethroids to bednets, an increasingly common procedure in modern malaria control regimes as reviewed by Rozendaal (1989) and Curtis *et al.* (1990). In such cases contact time is far shorter than the WHO standard exposure of one hour, with knockdown and kill within the first seconds or minutes in susceptible individuals (Hossain & Curtis, 1989). In

an attempt to make the tests more realistic, particularly when investigating sublethal pyrethroid effects on disease transmission induced by contact with impregnated nets, it was considered better to dispense with the standard WHO methodology. Thus, in the present project many of the experiments on *Plasmodium yoelii* development have been carried out by combining insecticide contact on a net with feeding on an infective host, because this is considered to provide a more accurate representation of events under user conditions.

2.2. Materials

2.2.1. Mosquitoes

ZANU *Anopheles gambiae* Colonised in London (LSHTM) in 1982. Eggs from wild caught females from Zanzibar, Tanzania

ZANDS *Anopheles gambiae* Colonised in London (LSHTM) in 1984. Mass selection from ZANU strain for DDT resistance. Also exhibits some dieldrin resistance

BEECH *Anopheles stephensi* A sub-colony from Beecham's laboratories. Originally collected in the field from New Delhi, India in 1947. Shows low levels of resistance to DDT. High infectivity with *Plasmodium* sp.

DUB/APR *Anopheles stephensi* A highly permethrin resistant strain selected in the laboratory from field material collected in Dubai by Ladonni & Townson (Ladonni, 1988)

ST MAL *Anopheles stephensi* Colonised in London (LSHTM) in 1979 from Lahore, Pakistan. Exhibits strong malathion specific resistance due to a qualitative change in carboxylesterase inherited as a single semi-dominant autosomal gene (Hemingway, 1983)

FEST *Anopheles albimanus* Sub-colony (LSHTM) 1981, from colony held at the Insect Affecting Man Laboratories, Gainsville, Florida. Wild collections from Fernando, El Salvador, 1975. Multiple resistance to organophosphates and carbamates due to an altered acetylcholinesterase target site (Hemingway *et al.*, 1986)

2.2.2. Insecticides

Permethrin 0.25 % on paper at a spreading rate of 3.6 g/m^2.

Supplied by WHO for use with standard bioassay tubes

Deltamethrin 99 % Technical. Supplied by ICI, Jealotts Hill.

Internal ref. ERH 379.88 JH

Lambda-cyhalothrin 88.6 %. Supplied by ICI, Jealotts Hill.

Internal ref. ERH 379.88 JH

Malathion 96 % technical (Fyfanon) supplied by A/S Cheminova

Ref. DK-7602 Lemvig

Propoxur 99 % technical. Supplied by National Physical

Laboratories, Teddington, Middx, UK. Cat No. p16-28, sample No. R0707

2.2.3. Chemicals

Silicone oil Shell (UK) Ltd, Carrington, Urmston, UK

2.3. Methods

2.3.1. Mosquito rearing

All stock mosquito colonies were maintained using the standard techniques employed by LSHTM for anopheline mosquitoes and detailed in "A general working manual and code of practice for the safe management of mosquitoes" (Sawyer, 1983). To ensure that environmental variation was minimised between tests and all replicates the following method of rearing all individuals for experiments was employed throughout :

From a single batch of eggs from the parent colony, 200 newly hatched larvae were placed in a white plastic bowl containing 2 liters of tap water, with a depth of around 6 cm. A small piece of turf was added to each bowl and the growing larvae were fed a proprietary powdered baby food, 'Farex®' each day and maintained at 25°C ± 2°C with relative humidity between 75 and 80 % controlled by heated water tanks regulated by humidistats. As adults emerged they were divided randomly into two groups, one group for treatment the other being the control, by collecting alternate small batches by mouth aspirator from the holding / emergence cage.

2.3.2. Preparation of exposure papers

In those experiments utilising insecticide treatments on impregnated papers the following standard preparation technique was employed. By dilution of the technical grade insecticide sample with a suitable oil base, a working solution was made with a concentration equal to the required percentage on the exposure paper. For example, to produce a 5 % malathion paper the technical grade (98.6 %) malathion was diluted with olive oil to give a working solution of 5 %. A volume of 0.7 ml of this working solution

was then decanted into a small glass bottle and 1.3 ml acetone was added as a spreading agent and the oil and acetone were mixed thoroughly. This total volume of 2 ml was then applied evenly over the entire surface of a piece of Whatman's No. 1 filter paper measuring 12cm x 15 cm. Applying this volume to this surface area gives the required spreading rate of 3.6 g/m² at the predetermined concentration, as recommended by the World Health Organisation (WHO, 1963). After hanging overnight to allow the acetone to evaporate, the papers were wrapped in aluminium foil and stored at 4°C when not in use.

2.3.3. Mosquito bioassay test kits

The standard World Health Organisation adult insecticide test kits were employed as described in WHO (1963). Mosquitoes in the treatment group were exposed to pyrethroid impregnated papers either supplied by WHO or made fresh prior to each experiment following the method described above (2.3.2.). Having placed about 25 individuals aged two - three days post emergence in a plastic tube lined with untreated paper, this non-insecticidal (resting) tube was screwed, via a sliding connecting plate, onto an exposure tube lined with insecticide impregnated filter paper. The mosquitoes were gently blown into the exposure tube and allowed to rest on the treated surface for a set period. The mosquitoes were then transferred back into the resting tube, given 10 % glucose solution via a cotton wool plug and left for 24 hours.

2.3.4. Choice of sublethal dose

Using the WHO test kits lined with pyrethroid paper as described above, rates of exposure were varied by increasing or decreasing the contact time, rather than by using several different doses. Exposure times of 5, 10, 15, 30, 45, 60 and 75 minutes were used, with any knockdown recorded at the end of these periods. After 24 hours, final mortality figures were recorded and are presented in tables 1 - 3. Between eight and ten replicates were conducted on each dose. Mortality at each time-point were entered into a computer-

based log probit analysis program (origin unknown but used historically at LSHTM and other Institutes, Curtis C.F., Personal Communication). This program computes the linear regression of probit mortality on log dose, with weighting for sample size, and derives the intercepts on the log dose axis corresponding to a specified mortality level, in this case, the LD_{50}.

2.3.5. Sublethal treatment with pyrethroids

Sublethal exposure in those experiments requiring contact with impregnated papers was achieved in a very similar way to that set out in 2.3.3. In the case of pyrethroids, however, silicone oil was used, rather than olive oil as was the case for organophosphates and carbamates. The exposure time in this case was that determined from the results obtained in the contact time / mortality study detailed in 2.3.4. and recorded in tables 1-3. After the 24 hour recovery period, mortality was recorded and the survivors then used in the subsequent experiments. The control group was treated in the same way but the exposure tube in the WHO insecticide test kit was lined with a silicone oil impregnated paper without pyrethroid. Based on normal WHO insecticide testing guidelines, an exposure of 1 hour on such papers would be used to determine resistance in mosquito populations, a period which would be expected to result in 100 % mortality in susceptible populations. The chosen sublethal dose derived from a treatment time of 15 minutes did not lead to knockdown during the exposure period, therefore the contact time with the insecticide can be considered to be consistent between individuals.

2.3.6. Impregnation of netting

Pyrethroid insecticides were impregnated onto polyester netting of 100 denier, as supplied J.C. Small & Tidmas Ltd or The Siam Dutch netting Co. This is of a type commonly used in the manufacture of bednets, which are undergoing field trials and operational usage with pyrethroid impregnation around the world. A piece of netting of 1.0 m^2 was weighed dry,

immersed in water, to become fully saturated, then removed and gently wrung to expel excess water. By re-weighing and subtracting the dry weight from the wet weight, the total volume of water which is retained per unit area of the material was ascertained. With this information, and for each desired final deposit density per unit area for each particular pyrethroid, it was possible to calculate what dilution of emulsifiable concentrate (E.C.) was required to impregnate each net sample. Each emulsion was made up with excess volume and net samples dipped, gently wrung to remove surplus liquid and left spread flat on aluminium foil overnight to dry, and stored in foil at 4°C whenever not in use.

2.4. Results

Twenty four hour mortality figures at each exposure time are presented in tables 1 to 3 for permethrin, deltamethrin and lambda-cyhalothrin respectively. Results are expressed as number dead / number tested, for example, 50 / 100 would indicate 50 % mortality. In each case the tables are followed by the LT_{50} derived using log probit analysis as detailed in 2.3.4.

Table 1. **Effect of varying exposure time of ZANU strain of *An. gambiae* to 0.25 % permethrin papers.**

Exposure Time (minutes)	Mortality (dead / total)	% Mortality
5	22/217	10.1
10	79/238	33.2
15	121/251	48.2
30	187/224	83.5
45	202/212	95.3
60	219/219	100.0
75	100/101	99.0

Log time probit mortality analysis

LT_{50} = 14.0 minutes

95 % CL of LT_{50} = 13.0 – 14.9

Heterogeneity χ^2 (5 d.f.) = 15.5 p= > 0.05 not significant.

Table 2. **Effect of varying exposure time of ZANU strain of *An. gambiae* to 0.025 % deltamethrin papers.**

Exposure Time (minutes)	Mortality (dead / total)	% Mortality
5	46/230	20.0
10	90/245	36.7
15	136/241	56.4
30	193/226	85.4
45	214/230	93.0
60	245/246	99.6
75	228/228	100.0

Log time probit mortality analysis

LT_{50} = 12.1 minutes

95 % CL of LT_{50} = 11.2 – 12.9

Heterogeneity χ^2 (5 d.f.) = 23.4 p= > 0.05 not significant.

Table 3. Effect of varying exposure time of ZANU strain of *An. gambiae* to 0.025 % lambda-cyhalothrin papers.

Exposure Time (minutes)	Mortality (dead / total)	% Mortality
5	35/225	15.6
10	101/250	40.4
15	141/238	59.2
30	203/230	88.2
45	225/235	95.7
60	224/224	100.0
75	185/185	100.0

Log time probit mortality analysis

LT_{50} = 11.8 minutes

95 % CL of LT_{50} = 11.0 – 12.6

Heterogeneity χ^2 (5 d.f.) = 12.6 p= > 0.05 not significant.

Summary of results

Permethrin LT_{50} 14.0 minutes (13.0 – 14.9)

Deltamethrin LT_{50} 12.1 minutes (11.2 – 12.9)

Lambda-cyhalothrin LT_{50} 11.8 minutes (11.0 – 12.6)

2.5. Discussion

It is anticipated that by using the standardised environmental rearing conditions detailed above for all insects used in this study it will be possible to minimise any inter or intra-replicate errors within or between individual experiments. Likewise, the choice of a fixed exposure period for sublethal doses to be administered of 15 minutes on WHO papers, and following identical insecticide impregnation procedures, should ensure minimal variation within each study.

CHAPTER 3 Effects on Adult Longevity

3.1. Introduction

Quite early in the development of insecticides it was reported that they could have an effect on longevity not directly related to the initial mortality. Schwartz (1951) reported 'latent toxicity' after treating adult *Leptinotarsa decemlineata* with DDT or BHC, which led to an initial 40 % mortality, and much later resulted in a significant extra proportion of over-wintering adults dying compared with the untreated controls. It is clearly essential to identify what is meant by the term 'sublethal effect' or 'latent toxicity' and not to confuse these with the 'acute' poisoning culminating in near immediate death post treatment. Moriarty (1969) suggests that where time of death following contact with pesticides is found to have a polymodal distribution, the initial phase should be defined as acute toxicity and any subsequent toxic phases as latent. Work on the housefly, *Musca domestica*, with a number of different insecticides have shown reduced longevity following sublethal exposure. Hunter *et al.* (1959) found a significant reduction following DDT contact, and Ouye & Knutson (1957) observed a similar phenomenon working with the organophos-phate, malathion. Following topical application of low doses of DDT, Adkisson & Wellso (1962) reported a reduced mean lifespan of up to 50 % in *Pectinophora gossypiella*, the Pink Bollworm moth. This group also observed an indirect effect on longevity in females which, when mated with sublethaly exposed males, actually lived longer than controls, as the mating ability of treated males was impaired and their mates began laying eggs later in life than normal. DDT has also been shown to reduce longevity in Mediterranean fruit flies, *Drosophila melanogaster*, and melon flies, *Bactrocera cucubitae*, following sublethal exposure in a study by Sherman (1958), whose work a few years later with resistant and susceptible strains of *M. domestica* found similar effects (Sherman & Sanchez, 1964). Diflubenzuron has been found to significantly reduce longevity of the bean bug, *Riptortus clavatus*, from 42.3 ± 12.7 days to 16.9 ± 2.9, although Kim *et al.*

(1992) reported this only to be true when treatment took place within the first few hours post emergence. Although not commented upon by these authors it would seem that either some hormonal or other physiological process in very young adults was being disrupted or alternatively, once the cuticle of the newly emerged adult began to harden, insufficient penetration of the insecticide occurred to affect longevity. Larvae of *Culex quinquefasciatus* exposed to LC_{50} levels of malathion, propoxur, resmethrin or methoprene were found to have a significant increase in development time prior to emergence (Robert & Olson, 1989). Such an effect in the field could be expected to decrease chances of larval survival by increasing time exposed to predators or habitat loss. Reyes-Villanueva *et al.* (1990) working on the larvae of *Aedes aegypti* treated with temephos, found that emerging adults actually lived longer than control females. The same experiments found that these treated females laid fewer egg batches than controls and, although not stated by the authors, these two factors are presumably related. That is to say that the increased longevity is likely to be a direct result of a reduction in the stressful activity of egg production and oviposition. As well as regular insecticidal compounds which have been shown to exert such changes on insect physiology, a number of other compounds and natural events can have similar ramifications. An imbalance of natural insect hormones, for example those mimicked by the insect growth regulator methoprene, can also effect longevity. Sawby *et al.* (1992) reported a significantly reduced adult lifespan in *Ae. aegypti* following larval exposure, with a correlation between increased dose and subsequent shortening of life expectancy in females. The effect on males was much less pronounced and possibly masked by the fact that this sex had a far lower lifespan even in the control group. An initial hypothesis of the authors that such effects were brought about by a reduction in glycogen availability was discounted during the study, and the only conclusion drawn was that 'neuroendocrine abnormalities' were contributing to the insect's early death. This study corroborated the findings some 5 years earlier by Bouchard & Wilson (1987) in which juvenile exposure to methoprene resulted in reduced

longevity of adult *D. melanogaster*. Natural infections of pathogens and parasites have also been shown to be detrimental to the longevity of mosquitoes. Spores of the microsporidian *Nosema algerae* ingested at any larval stage of *Anopheles albimanus* have been shown by Anthony *et al.* (1978) to sharply reduce adult lifespan. It was concluded that, in this important vector species, the observed halving of time at which 90% of a cohort had died in treated females would reduce the number of potential sporozoite infective individuals in a population by between 85 and 97 %. Klein *et al.* (1982) compared cumulative mortality rates between control *An. dirus* and those infected with *Plasmodium cynomolgi*. Survival was identical through days 1 - 10, after which there was a marked increase in mortality of the infected group. Postmortem dissection of fresh corpses of infected females between day 9 - 20 revealed accumulations of bacteria and tissue deterioration in the midgut and salivary glands, almost certainly due to physical damage caused by rupturing oocysts and sporozoite invasion. Similar effects of reduced longevity and fecundity are reported by Hogg & Hurd (1997), in a range of *Plasmodium* infected anophelines.

3.1.1. Aims of this chapter

Having reviewed the importance and factors affecting the longevity of medically important insects, this study was designed to establish the effects of sublethal exposure of pyrethroids on the malaria vector *Anopheles gambiae*. Groups of exposed and untreated control adults were observed daily and mortality was recorded. Data were analysed to produce comparative life tables and differences are compared between treated and untreated groups. The findings are discussed in terms of potential effects on disease transmission and vector control methods commonly employed.

3.2. Materials

3.2.1. Mosquitoes

ZANDS *Anopheles gambiae*

3.2.2. Insecticides

Permethrin	0.25 % on paper
Deltamethrin	0.025 % on paper
Lambda-cyhalothrin	0.025 % on paper

3.3. Methods

The day after emergence, mosquitoes in the treatment group were exposed to pyrethroid impregnated papers, either supplied by W.H.O. or made fresh prior to each experiment, following the method described in chapter 2.3.2 above. The interconnecting plastic bioassay tubes were then utilised as recommended in the standard W.H.O. procedure (WHO, 1963a) After exposure for 15 minutes (the standard sublethal dose determined in chapter 2) the mosquitoes were transferred to recovery tubes and given glucose solution via a cotton wool plug and left for 24 hours. After the recovery period, mortality was recorded and the survivors then used in the subsequent experiments. Based on normal WHO insecticide testing guidelines (WHO , 1963a&b) an exposure of 1 hour would normally be used to determine resistance in mosquito populations, a period which would be expected to result in 100 % mortality in susceptible populations. The chosen sublethal dose, derived from a treatment time of 15 minutes, did not lead to knockdown during the exposure period with any of the pyrethroids used, so the contact time with the insecticide can be considered to be consistent between individuals. The control group was treated in the same way but the exposure tube in the WHO test kit was lined with paper impregnated with silicone oil without pyrethroid.

3.3.1. Analytical methods

When measuring survival of a cohort it is important to not only consider when death of an individual occurs, but also how long it had survived before death. To do this, Kirkwood (1988) recommends 2 different, but complimentary approaches. Firstly, survival curves

for the comparative treatment groups are plotted visually, then statistical significance of any differences observed can be assessed using the log rank test. This test, which is a special application of the Mantel-Haenszel Chi square test, constructs a separate 2 x 2 table for each time interval which compares the proportions of treated and control mosquitoes dying during each. In this case it is used to compare the numbers dying each day with the numbers surviving / day in each group as follows :

	Numbers dying in a given day	Numbers surviving that day
Control group		
Treated group		

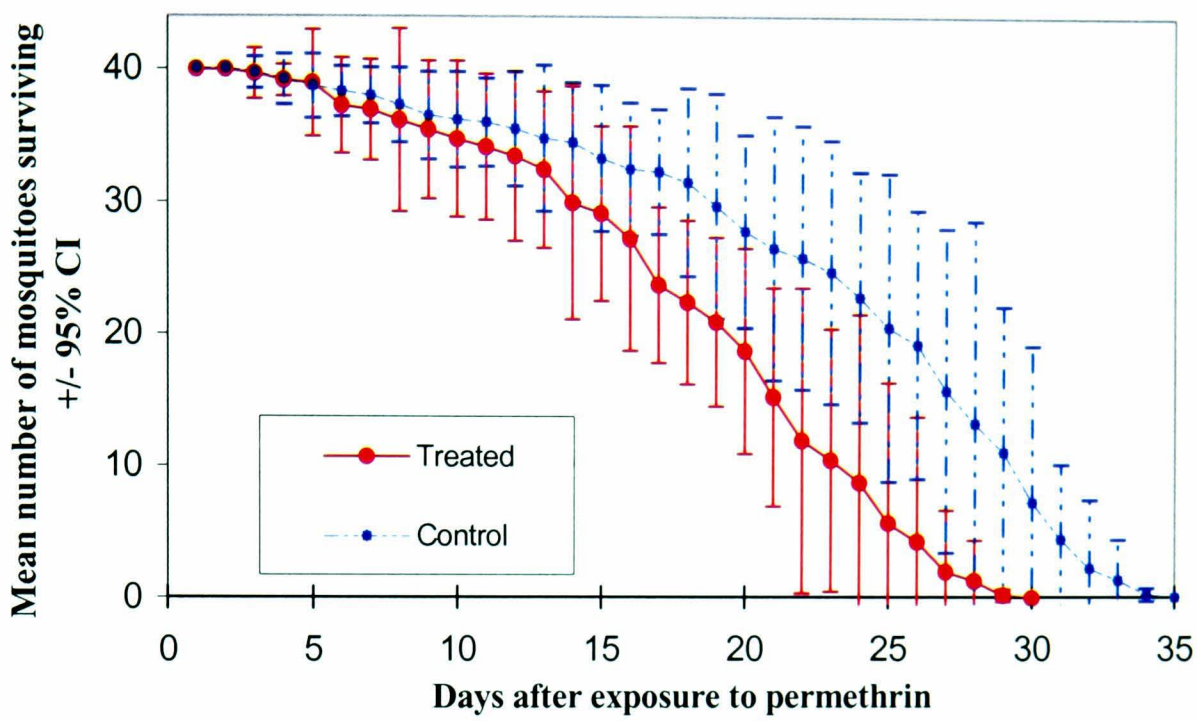
A separate 2 x 2 table is constructed for each day until there are no survivors in one or other group. Using the STATCALC facility of EPI INFO 6.0 the results from each daily table are accumulated by the Mantel-Haenszel procedure to give a single χ^2_{MH} with 1 degree of freedom. The corresponding *p* value is a measure of the statistical significance of the deviation of the mortalities of the treated and control groups.

3.4. Results

3.4.1. Permethrin

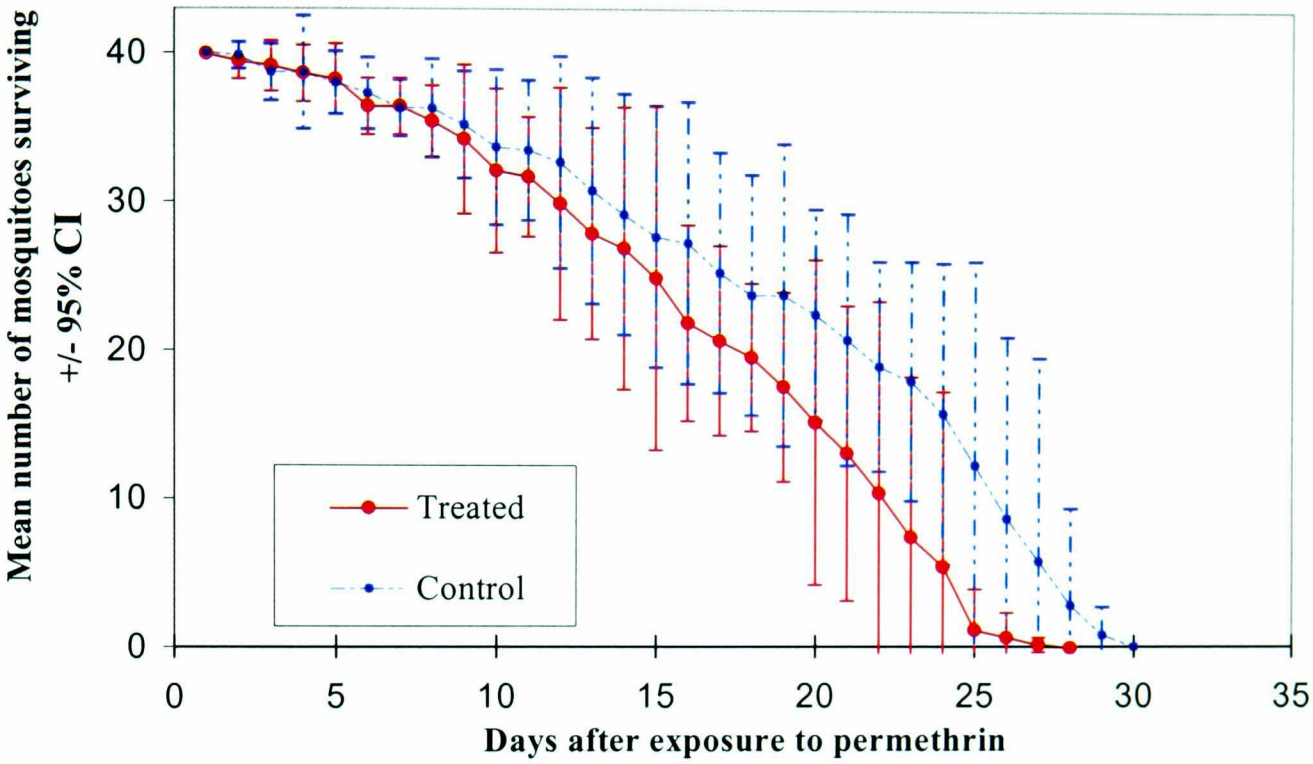
Figures 5 and 6 are life tables to show the effect of 15 minutes exposure to papers treated with 0.25 % permethrin on the longevity of females and males respectively. In all cases the following graphs show an average of 8 replicates of 40 individuals, are plotted with 95 % confidence intervals based on standard deviations between replicates and begin at day 2 post-exposure, to exclude the immediate mortality.

Figure 5. **Effect of permethrin exposure on *Anopheles gambiae* adult female longevity.**



Log rank test χ^2_{MH} = 71.10 (df=1) $p < 0.0001$ Indicating a significant reduction in survival of females following sublethal exposure to permethrin compared to controls.

Figure 6. **Effect of permethrin exposure on *Anopheles gambiae* adult male longevity.**



Log rank test χ^2_{MH} = 47.79 (df=1) $p < 0.0001$ Indicating a significant reduction in survival of males following sublethal exposure to permethrin compared to controls.

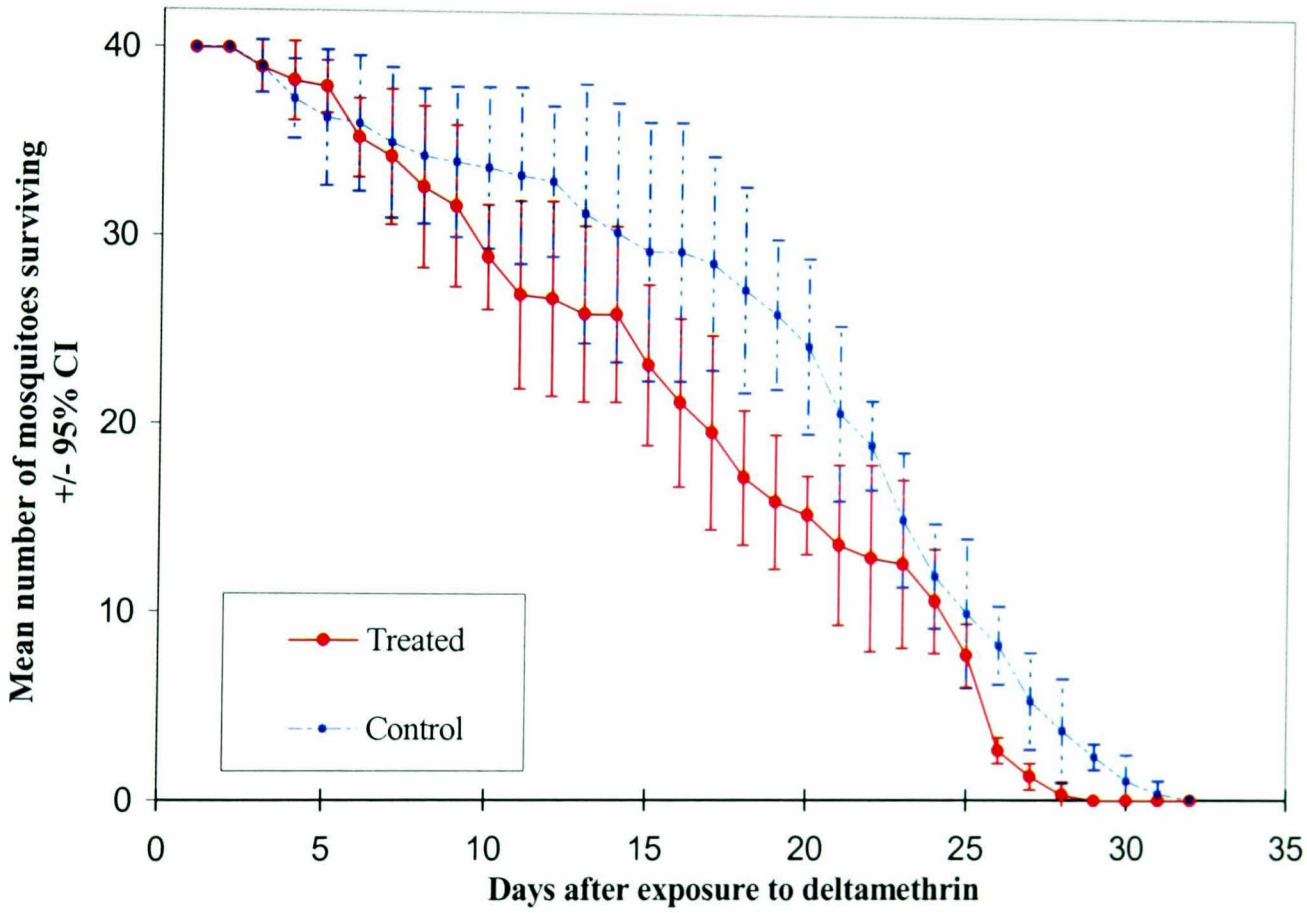
It is clear that in both sexes permethrin causes a significant reduction in longevity which appears not to be directly linked with the mean initial 48 % (n=8 replicates of 40 individuals) kill recorded in the 24 hours after insecticide contact. Over the first 5-10 days there is little difference between the control and treated groups but thereafter the survival curves diverge more markedly. This pattern of gradual increased mortality clearly fits the definition put by Moriarty (1969) as being truly a 'latent' sublethal effect rather than delayed mortality. As this phenomenon culminates in the unnaturally early death of the

insect it could be thought of as premature senescence as has been observed in irradiated insects (Langley & Curtis 1975).

3.4.2. Deltamethrin

Longevity of females exposed for 15 minutes to papers treated with 0.025 % deltamethrin compared with control individuals are shown in figure 7.

Figure 7. **Effect of deltamethrin exposure on *Anopheles gambiae* adult female longevity.**



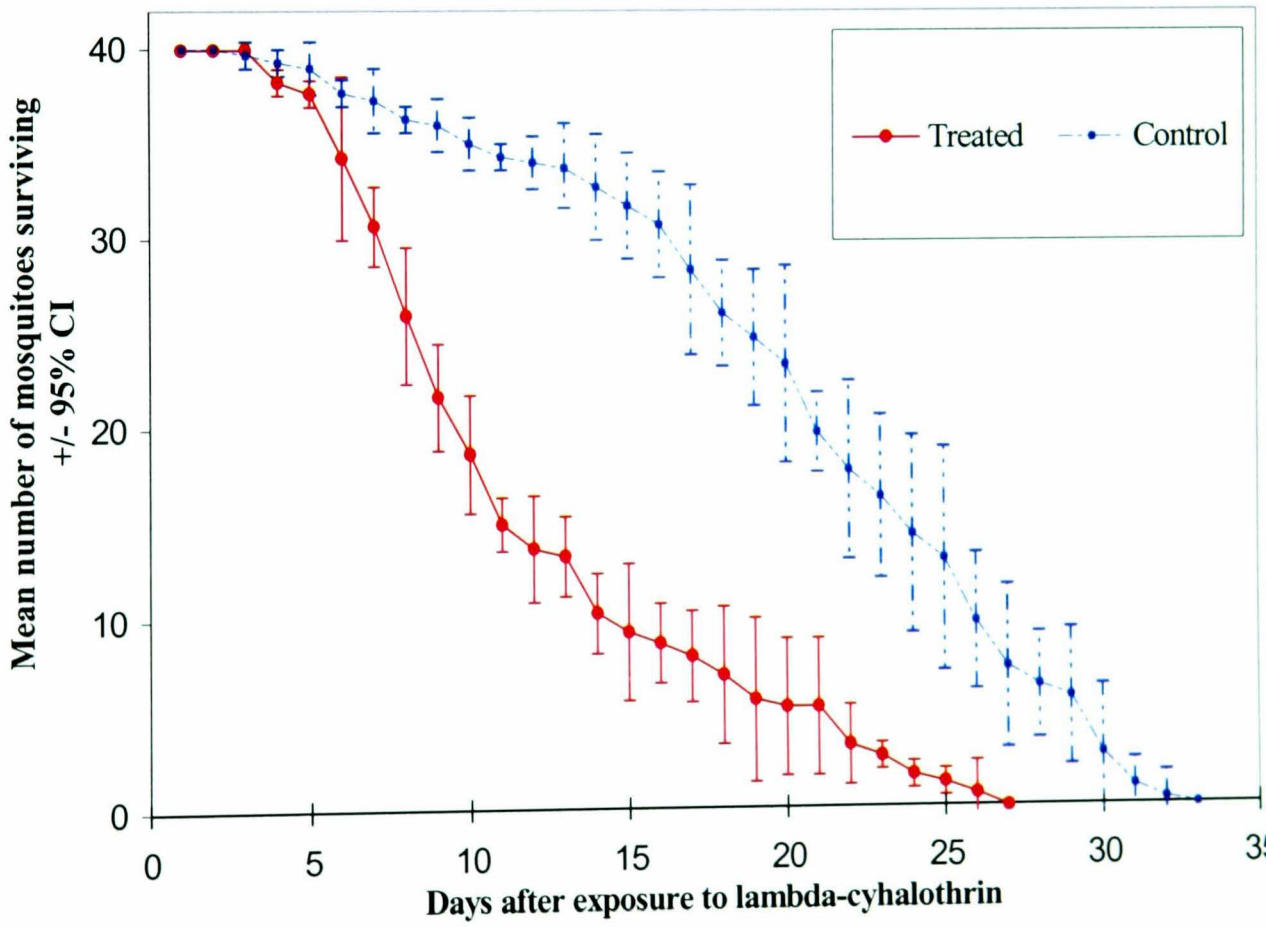
Log rank test χ^2_{MH} = 19.95 (df=1) p = 0.000008 Indicating a significant reduction in survival of females following sublethal exposure to deltamethrin compared to controls.

The mean immediate mortality was 56 % (n=8 replicates of 40 individuals) and once again there is a significant reduction in longevity following treatment as shown by the significant divergence of the survival curves, especially between days 10 and 20.

3.4.3. Lambda-cyhalothrin

15 minute exposure to 0.025 % Lambdacyhalothrin caused a mean (n=8 replicates of 40 individuals) mortality of 58 % in the first 24 hours. Thereafter a very marked delayed effect on female longevity was evident, as shown in figure 8.

Figure 8. **Effect of lambda-cyhalothrin exposure on *An. gambiae* adult female longevity.**



Log rank test χ^2_{MH} = 97.09 (df=1) $p < 0.0001$ Indicating a significant reduction in survival of females following sublethal lambda-cyhalothrin exposure.

In addition to the gradual increase in mortality with time as observed with both permethrin and deltamethrin there appears to be a more pronounced mortality approximately one week post treatment with lambda-cyhalothrin. This implies that as well as the premature senescence already described, this compound has either an additional delayed toxicity or has triggered some chain of physiological events resulting in yet further accelerated aging.

3.5. Discussion

The daily survival rate of adult mosquitoes is a crucial factor in their ability to transmit disease, and is an integral part of any consideration of control or eradication programmes based on adulticides. Male survival is relatively less important, in many respects, than that of the females, as a female often only mates once in her lifetime, and males are capable of inseminating several females (Gomulski, 1988). The longevity of females is a critical factor in the theory and measurement of disease transmission and its epidemiology, with only a small percentage of females living long enough to pick up an infection, complete the extrinsic cycle and pass on the infection. Lines *et al.* (1990), measured sporozoite rates in wild Tanzanian *An. gambiae* whose ages were simultaneously determined using Polovodova's technique (Detinova, 1962). This study reported that extremely high sporozoite rates (>50 %) were found in the oldest females, thus emphasizing the potential benefits expected from reducing longevity. The same study calculated an average daily survival rate of females in the field to be 81.9 %, similar to that reported by Fernandez-Salas *et al.* (1994) of 87 % in *An. pseudopunctipennis* from Mexico. A more recent study in South East Tanzania by Takken *et al.* (1998b) utilised the mark-release-recapture technique with wild *An. funestus* and *An. gambiae*. This study derived an estimated daily survival rate of 63 % and 78 % respectively based on regression of the recapture rate on time since release. These rates are comparable with recent data from Southern Mexico presented by Arredonondo-Jiménez (1998) of 68 % in forest dwelling *An. vestitipennis*, although this dropped to 45 % - 58 % in coastal areas. Macdonald (1957) concluded that

under field conditions the principle causes of death in mosquitoes are 'the hazards of daily life' and that an individual's survival probability each day was independent of its age. However, retrospective analysis of a number of earlier field studies by Clements & Paterson (1981), suggest there is higher mortality in older individuals in many species of anophelines, and that the increase in daily probability of death occurs in a logarithmic manner directly proportional to age. Which ever of these is the case, any additional reduction in the life expectancy may be expected to have a significant effect on a malarial vector's ability to transmit disease. The mosquito component of the basic reproduction rate of malaria is known as vectorial capacity. Service (1993) defines vectorial capacity as the average number of innoculations from a single case of malaria in unit time (usually per day) that the vector population transmits to man, assuming that all insects feeding on a gametocyte carrying host become infected. This parameter is the fundamental method of assessing potential for disease transmission by the vector population and has been expressed in a variety of mathematical models over the years, the most utilised of which are discussed by Service (1993). Whatever model is used, one of the most important variables which influence vectorial capacity is female longevity, or survival rates. A frequently used formula derived by Garrett-Jones (1964) is;

$$C = \frac{ma^2 p^n}{-\log_e p}$$

Where C = vectorial capacity, ma = the number bites / man / day, p = the probability of daily survival of a female and n = time for a female to develop infective sporozoites. Clearly, the longevity of a mosquito is a very important factor in determining likelihood of transmission, not only does it influence whether or not an individual is capable of completing extrinsic incubation of the malarial parasite to become infective, but also the

number of potential hosts it subsequently inoculates. Birley & Boorman (1982) derived the following equation to estimate the infective life of a vector (V) ;

$$V = p^{d/u} / (1 - p)$$

Where p = survival rate per oviposition cycle, d = the duration of incubation period and u = estimated period between feeding and oviposition. When expressed thus it is clear that the infective life of a mosquito, V, is extremely sensitive to even small changes in the survival rate, p.

In this work under laboratory conditions, with the mosquitoes being provided with optimal conditions and isolation from predators, the individuals could be expected to live longer than under natural conditions, with mortality heavily influenced by senescence, i.e. increased probability of death at greater ages. Although the consequences of these rather artificial conditions is a relatively longer period for any sublethal effects to be detected, it may also be true that such effects would occur sooner in the field where the individuals are under greater stress or higher physical risk, for example, during host feeding or oviposition. The late stage in the life span when the difference in survival is most noticeable indicates that this is truly premature senescence, rather than simply a delayed mortality caused by the original toxic effects of contact with the insecticide. The exact cause of the phenomenon is difficult to surmise but is likely to be a direct consequence of damage caused to the insect physiology by the disruption of the nervous system and associated aberrations due to abnormal hormone release and dehydration which may also occur. The significant reduction in adult longevity can be seen as an additional benefit compounding the effect of the direct mortality due to the use of an insecticide, and should be taken into consideration when evaluating such control measures with pyrethroids and in particular the use of impregnated bed nets. Magesa *et al.* (1991) report evidence from

ovarian dissections that *An. gambiae* in Tanzanian villages where the people use treated nets have reduced longevity, and it would be interesting to investigate whether sublethal effects could by playing a part in this or similar field situations, when it has been shown that widespread use of treated nets reduce the sporozoite rate.

CHAPTER 4 Effects on reproductive potential

4.1. Introduction

A wide range of insecticides have been reported to have a variety of effects on the number and viability of offspring produced by insects surviving exposure. Moriarty (1969), points out the fact that such changes may not just be due to direct interference in, or stimulation of, the process of oogenesis, but may also be brought about by indirect means, for example reduced feeding efficacy or variation in mating behaviour.

4.1.1. Effects on egg development

The most straightforward effect is reported by Beard (1965), who found that DDT in the diet of *Musca domestica* at levels inducing 'negligible mortality' caused inhibition of ovarian development and a 50 % reduction in number of eggs laid, compared to a control group. Developmental inhibition of ovarioles was also the conclusion drawn by Kim *et al.* (1992) when they found that exposure of *Riptortus clavatus* to very low levels of diflubenzuron resulted in reduction of egg production by at least 75 %, and often cessation of egg production altogether. Derbeneva-Uhova *et al.* (1966), observed pathological aberrations in the ovaries of *M. domestica* and *Protophormia terraneovae* in females surviving DDT treatment during a programme of artificial selection for resistance. Sutherland *et al.* (1967) reported over 30 % increase in the number of ovarioles with follicles within females produced from *Aedes aegypti* larvae exposed to DDT. It is possible, however, that this may have been due to higher mortality of the smaller larvae, and thus selection for larger emerging females which could subsequently produce larger batches of eggs. Walker (1970) also found an increase in the number of follicles, but in this case the effect was seen in the F1 generation of *M. domestica* treated with DDT as larvae. DDT has also been found to reduce egg production in *Tribolium confusum* (Loschiavo, 1955), *Drosophila melanogaster* (Tattersfield & Kerridge, 1959) and

Pectinophora gossypiella (Adkisson & Wellso, 1962). Singh & Lal (1966) used a range of doses of DDT and found that the effects were very different on red cotton bugs (*Dysdercus fasciatus*), which laid more eggs after low dose treatments but at higher doses laid fewer eggs which also showed reduced hatch. As well as DDT, a number of other insecticides have been found to significantly reduce egg production. Georghiou (1965) applied an LD_{10} dose of a range of carbamates and observed a large decrease in the number of eggs produced by *M. domestica* of between 24 % - 51 %. An interesting series of experiments were conducted by Gardner *et al.* (1993) and Tesh & Guzman (1990), where a range of mosquito species were fed on dogs or humans undergoing ivermectin treatment. In both studies, all the surviving females produced 32 % fewer eggs which subsequently showed reduced hatch rate of between 40 % - 50 % of control levels. Larvae of the mosquito *Ae. aegypti* exposed to temephos were observed by Reyes-Villanueva *et al.* (1990) to have reduced fecundity as adults. Not only did mean egg production fall by between 37 % and 69 % in the treated group but they also stopped producing eggs earlier in life compared to the controls. The pyrethroid resmethrin, at low doses, was seen by Focks (1984) to significantly reduce the percentage egg production per female in the mosquito *Toxorhynchites rutilus* from 5.6 / female / day in controls, to 2.3 / female / day. Robert & Olson (1989) exposed *Culex quinquefasciatus* larvae to a range of insecticides with varying effects. Females treated with very low levels of malathion or methoprene gave slightly higher numbers of eggs and in larger raft sizes. However, at increased doses the reverse was true, with fewer eggs per raft (malathion 50 %, methoprene 39 %), smaller individual egg size and, in addition, the hatch rate also fell by 30 % in both cases. In the same experiments it was found that following exposure to either resmethrin or propoxur the proportion of females in the emergent population fell, but conversely this proportion was increased by malathion. In all cases there was also a recorded increase in the development times of emerging larvae. Exactly how egg development might be affected by pesticide exposure has yet to be elucidated fully, although the most likely mode of action is

disruption of the regulatory hormone control. It may be interesting, for example, to use methods similar to those employed by Jahan & Hurd (1998), where an immunological assay was developed to quantify vitellogenin and vitellin to investigate egg reduction in *An. stephensi* infected with *Plasmodium yoelii nigeriensis.*

4.1.2. Indirect effects on fecundity

As well as direct effects on egg development there are other factors which can bring about a change in reproductive potential, for example, mating efficacy, feeding behaviour, egg sterility and viability of offspring. Various studies have been carried out over the years which show all of these additional factors can be altered following sublethal insecticide contact. DDT has been found to reduce incidence of mating in *Pectinophora gossypiella* moths (Adkisson & Welsso, 1962). Kwan & Gatehouse (1978) reported that male *Glossina morsitans* treated with dieldrin or endosulfan were prone to premature separation during copulation which could impair insemination. Much work has been published on the effects of pyrethroids on the chemical and auditory mechanisms involved in mate recognition and attraction, almost all of it on crop pests, and is reviewed by Haynes (1988). In many species, deleterious effects have been observed on courtship using sound (Young & Stephen, 1970), the release and perception of pheromones (Floyd & Crowder, 1981; Haynes & Baker, 1985; Lucus & Renou, 1992), and the ability of sustained flight to a prospective mate (Linn & Roelofs, 1984), all of which will reduce mating efficacy. Feeding behaviour was observed in the tsetse fly, *G. morsitans*, 48 hours post treatment with dieldrin, endosulfan or permethrin by Kwan & Gatehouse (1978) and reduced bloodmeal size was recorded. Liu *et al.* (1986) also found significant reduction in blood engorgement, using a range of doses of three different pyrethroids in the mosquito *Ae. aegypti*, although the authors could offer no explanation of the mechanism for this depressive effect. One possible cause for such changes in behaviour was investigated by Chadd & Brady (1982), who recorded the effects of sublethal doses of permethrin on tsetse

flies and blowflies. Although DDT was seen to cause a temporary increase in the probing responsiveness, permethrin resulted in a prolonged and marked reduction in this primary feeding behaviour. A reduced incidence of leaf stippling associated with feeding of the plant pest, *Liriomyza trifolii*, was found to correlate to a reduction in oviposition following contact with abermectin by Schuster & Taylor (1988). Hodjat (1971) reported that, as well as reducing the number of eggs produced per female in *Dysdercus fasciatus*, DDT also resulted in a significant drop in their percentage hatch, and similar observations were made by Hunter *et al.* (1958 & 1959) in the house fly. In mosquitoes, De Coursey & Webster (1972) have reported reductions in fecundity, fertility and egg hatch following exposure of *Ae. aegypti* to a range of organochlorines.

4.1.3. Physical damage and fecundity

As well as the variety of physiological and behavioural factors detailed above, there is a well documented physical abnormality which is often brought about in adult mosquitoes which have survived low dose pyrethroid exposure. Khoo & Sutherland (1981) described this condition, known as 'leg fracture', which usually results in the loss of one or both hind legs due to excessive flexing of leg motor muscles. A similar effect has been attributed by Cox (1989), to the mode of action of formamidine pesticides on proleg aberrations in some caterpillar pests. It is possible that the hind legs of females play an active role in egg laying (Pile, 1989) and therefore not unreasonable to suggest such physical disfigurement may reduce egg laying efficiency. Diamondback moth adults, *Plutella xylostella*, were reported by Moore *et al.* (1992), to lose legs whilst walking over fenvalerate and were found to have a fecundity of only 48 % of their fully-limbed counterparts. In the same paper the authors suggest that this phenomenon is a fitness adaptation of the moth to reduce uptake of insecticide on contact. The subsequent loss in reproductive potential which results would presumably be outweighed in the field by faster recovery and lower mortality from residual sprays. Work by Liu *et al.* (1986) which showed that legs were lost by

female *Ae. aegypti* exposed to any of the 3 different pyrethroids they tested confirms this phenomenon to be a widespread effect of this group of insecticides. Eliason *et al.* (1990) comment upon this effect, stating that *Culex* mosquitoes sublethally exposed to resmethrin lost from one, to all of their legs, with many still able to fly and otherwise appearing unharmed. Bouchard & Wilson (1987) believed that at least part of the reason why a high proportion of male *D. melanogaster* were infertile following larval contact with methoprene was due to incomplete rotation of external genitalia, although such obvious physical deformity could not explain why similarly treated females produced fewer eggs when mated with untreated males.

4.1.4. Aims of this chapter

A wide variety of effects have been reported in a range of insect species following sublethal contact with many different classes of insecticides. This study aimed to investigate the effects of pyrethroid exposure on egg production per female per gonotrophic cycle, as well as total lifetime egg production per female. It also aimed to observe viability of eggs which were produced. Findings are discussed in relation to anticipated effects at the vector population level and possible impact on malaria transmission and vector control programmes.

4.2. Materials

4.2.1. Mosquitoes

ZANU	*Anopheles gambiae*

4.2.2. Insecticides

Permethrin	0.25 % papers
Deltamethrin	0.025 % papers
Lambda-cyhalothrin	0.025% papers

4.3. Methods

4.3.1. Egg laying

Female ZANU reared under standard conditions were divided into random groups and treated with the insecticides or an oil control, then placed in netting cages 15cm x 15cm x 15cm at a density of 10 ♀♀ per cage. To each cage, 10 untreated young male ZANU were added, and mating was allowed for 3 days before removal of the males. Following trial dissections of spermathicae from females after 3 days mating in this way it was ascertained that 100 % fertilisation could be expected. Females in each cage were offered a blood meal on an anaesthetised guinea pig twice each week, and 2 days after each feed an egg bowl lined with filter paper and containing water was supplied for oviposition. The surviving number of females was recorded before each egg bowl was added and, by counting the number of eggs, it was possible to calculate the mean number of eggs laid per surviving female. Each batch of eggs was kept for further experimental work. Successive blood meals were offered and all eggs were collected until all the females had died. A total of 6 replicates were conducted for each pyrethroid. Data were checked for normality using the Anderson-Darling test using Minitab for windows NT version 11.0, which fits the data to a normal probability plot and computes deviation from the expected straight line, with a high correlation signifying normality. Total lifetime egg numbers over the 6 replicates were compared for significance using a paired t-test, utilising the statistical package Minitab for windows NT version 11.0. The t-test compared differences in lifetime egg yield between treatment group and corresponding control group in each of the 6 replicates.

4.3.2. Percentage egg hatch

The percentage egg hatch from permethrin treated or control groups was determined as an extension of the procedure described above (4.3.1). After counting, the eggs were

maintained under standard rearing conditions and allowed to hatch for a period of 7 days, counting larvae each day. The resultant number of larvae were recorded to enable the percentage hatch to be calculated. Hatch rate was calculated from each of the 6 replicates using permethrin in 4.3.1 above. Egg hatch data expressed as a percentage of total egg numbers were arc sine transformed before the mean and 95 % confidence intervals were derived and results plotted graphically. Statistical significance of any differences between proportion of eggs hatching in the two groups was established by Mantel-Haenszel Chi square test as follows ;

	Proportion of eggs hatching	Proportion of eggs not hatching
Control		
Treated		

This test compares a series of stratified 2 x 2 tables without the need to pool data from individual replicates which might be invalid if there is heterogeneity between the results of each replicate.

4.3.3. Effects of leg fracture on oviposition

Leg fracture was simulated without the use of insecticide exposure by removing one or both hind leg(s) with fine entomological forceps close to the thorax. Three groups of mated ZANU females were selected and knocked down by chilling in a freezer for 3 minutes. One group had both hind legs removed and in another group only one hind leg was removed prior to placing the mosquitoes in a 15 x 15 x 15 cm cage to recover, whilst the control group was knocked down by chilling and placed with fine forceps directly into a second cage. After a 24 hour recovery period, equal numbers (12) of each group were offered a blood meal on an anaesthetised guinea pig, and on the second evening a receptacle for egg laying was provided. The eggs were counted and the mean number of

eggs per surviving female was calculated over a total of 6 replicates. Using the Minitab 11.0 for NT windows statistical package, the Anderson-Darling normality test was applied to check normality and a one way analysis of variance comparing the 3 groups (n=6) to determine significance.

4.4. Results

4.4.1. Effects of permethrin exposure on egg production

Figure 9 shows the average number of eggs laid per surviving female at each gonotrophic cycle ± 95 % confidence intervals (n=6). Whilst there is no obvious difference between the eggs laid by treatment or control groups up to cycle 8, it can be seen that fewer gonotrophic cycles were completed by treated individuals, who died earlier than those that were untreated.

Figure 9. **Effect of permethrin exposure on egg numbers laid per surviving female at each gonotrophic cycle.**

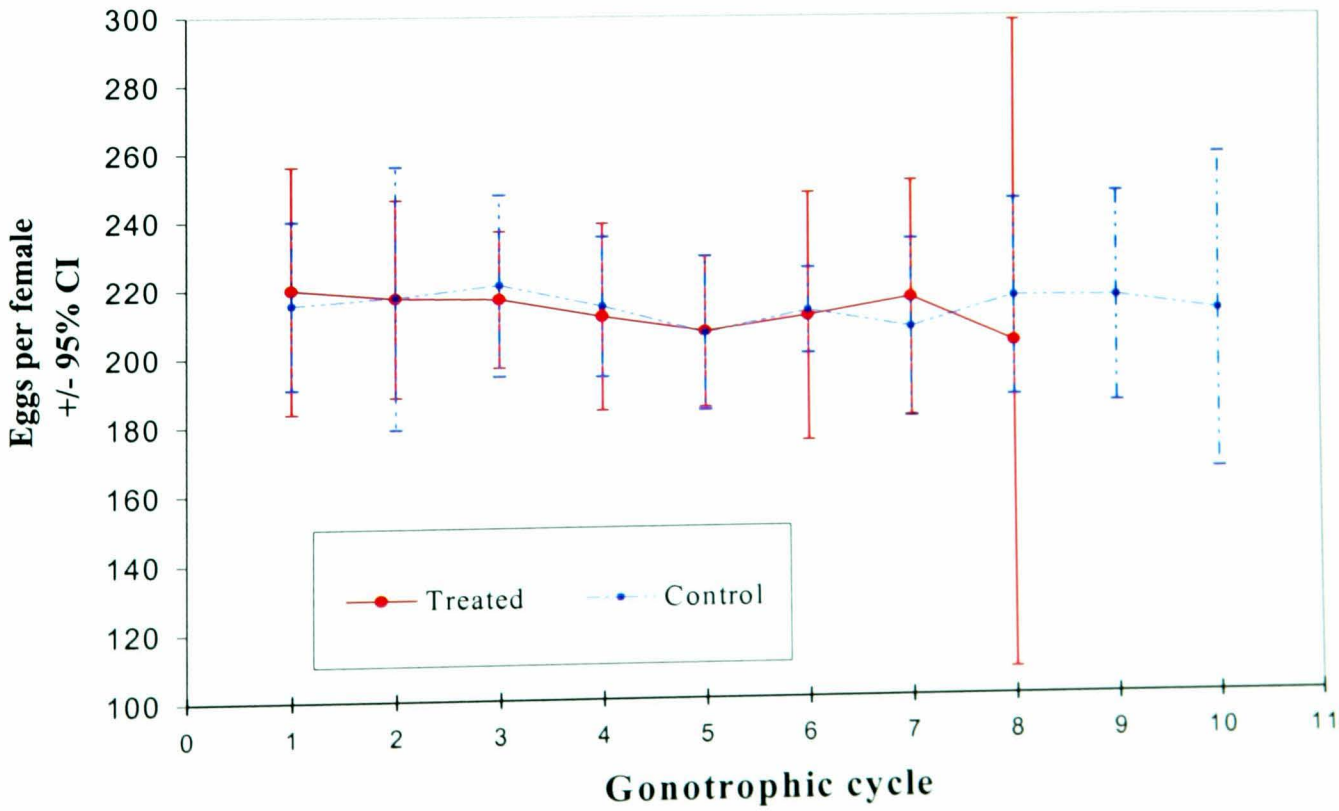


Table 4. **Lifetime egg production in each of the 6 replicates of 10 females - Permethrin**

Replicate	Control	Permethrin
1	15039	12185
2	12518	11983
3	16589	14446
4	15681	12088
5	15167	13516
6	14408	12860
Mean	14900	12846

Anderson-Darling normality test on the differences within each replicate between total lifetime egg production showed data were normally distributed in both control (p=0.513) and treatment groups (p=0.268)

Paired t-Test t=4.69 p=0.0054 (5 df) indicating a significant reduction in lifetime egg production in the permethrin treated groups compared to the corresponding controls.

Permethrin treated females were found to have a mean reduction (± C.L.) in total lifetime egg production of 13.98 % ± 6.2 % compared to controls.

4.4.2. Effects of deltamethrin exposure on egg production

Figure 10. **Effect of deltamethrin exposure on egg numbers laid per surviving female at each gonotrophic cycle.**

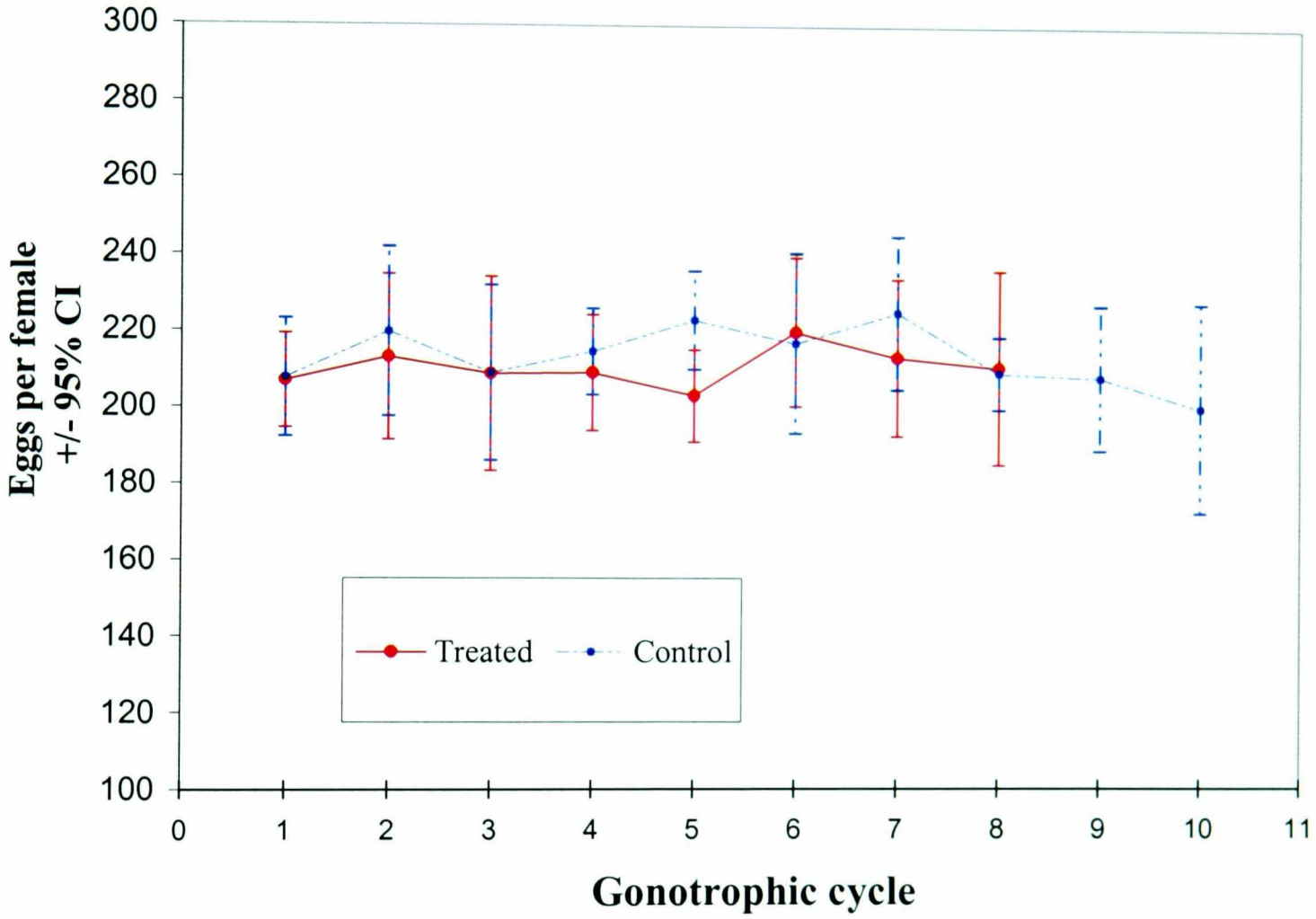


Table 5. **Lifetime egg production in each of the 6 replicates of 10 females - Deltamethrin**

Replicate	Control	Deltamethrin
1	12825	10789
2	12545	9158
3	14129	11381
4	13641	9717
5	14316	11002
6	12944	9413
Mean	13400	10243

Anderson-Darling normality test on the total lifetime egg production per replicate showed data were normally distributed in both control ($p=0.440$) and treatment groups ($p=0.358$).

Paired t-Test $t=11.58$ $p< 0.0001$ (5 df) indicating a significant reduction in lifetime egg production in females exposed to deltamethrin compared to the corresponding control groups.

Over the course of the 6 replicates there was a mean (± C.L.) 23.6 % ± 5.1 % reduction in total lifetime egg production in the deltamethrin treated group compared to controls.

4.4.3. Effects of lambda-cyhalothrin on egg production

Figure 11. **Effect of permethrin exposure on egg numbers laid per surviving female at each gonotrophic cycle.**

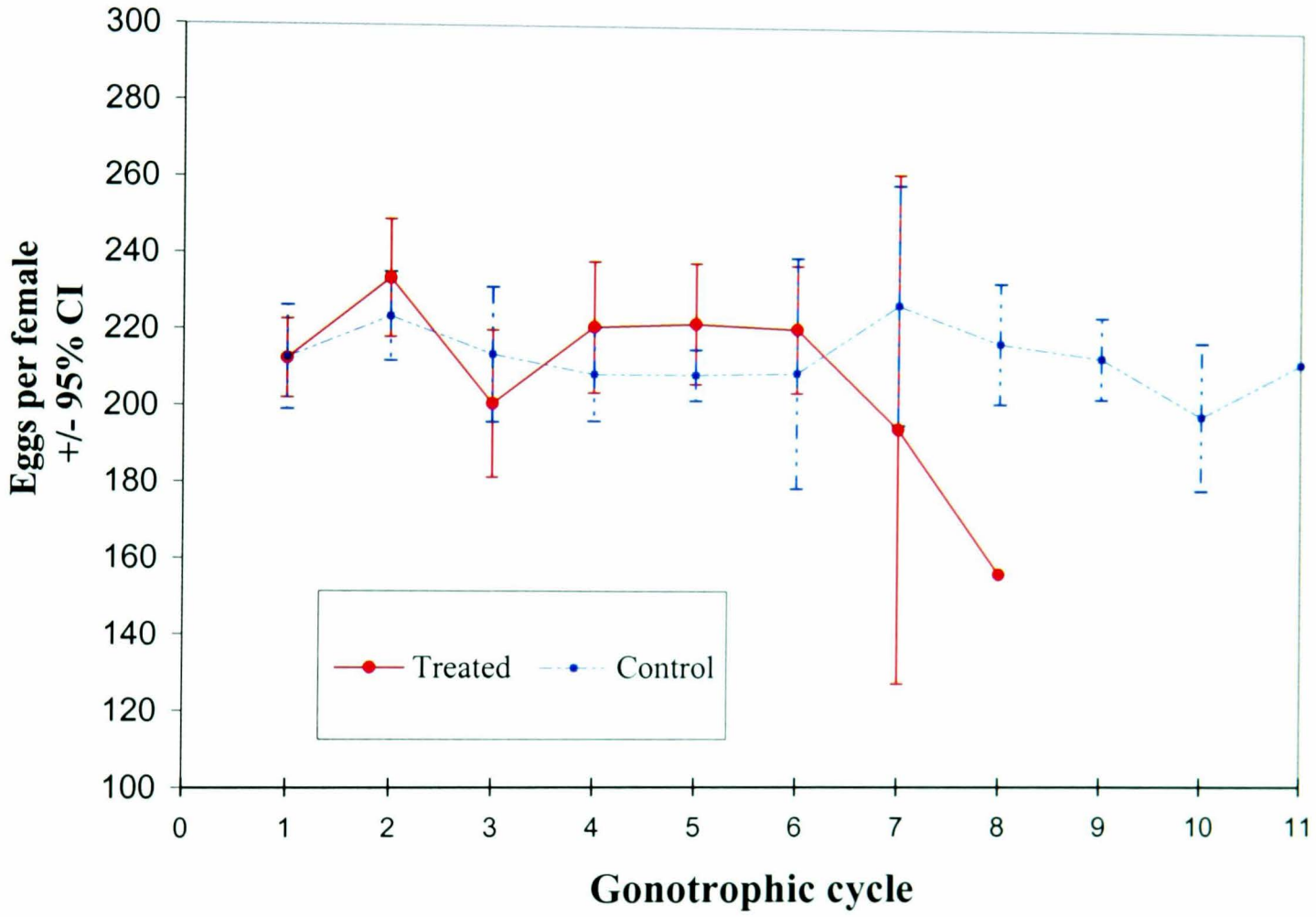


Table 6. Lifetime egg production in each of 6 replicates of 10 females – Lambda-cyhalothrin

Replicate	Control	Lambda-cyhalothrin
1	12857	10371
2	13631	9057
3	12277	9162
4	13036	10107
5	12886	9414
6	12763	9016
Mean	12908	9521

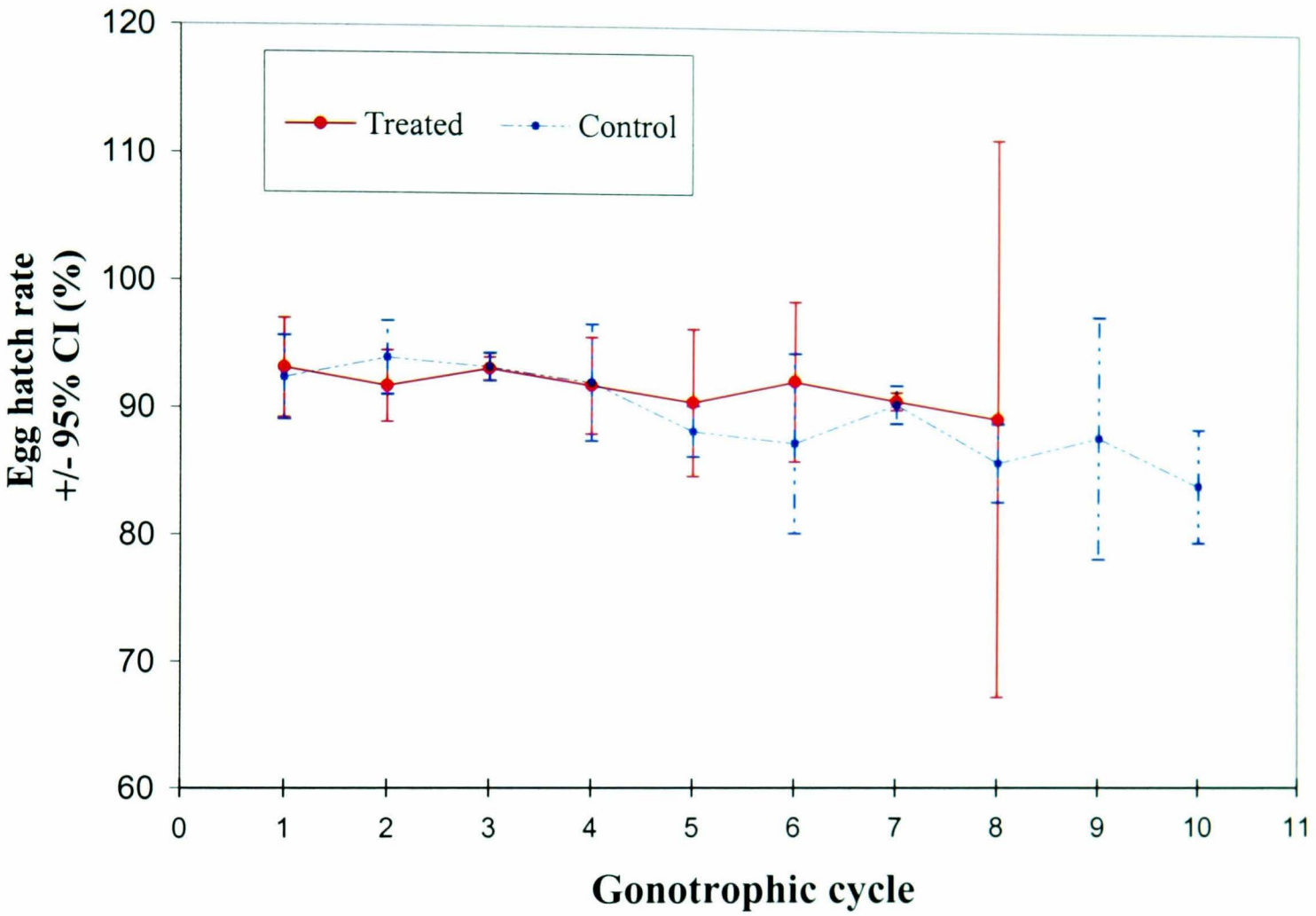
Anderson-Darling normality test on the total lifetime egg production per replicate showed that the data were normally distributed in both control ($p=0.376$) and treatment groups ($p=0.142$).

Paired t-Test $t=11.42$ $p < 0.0001$ (5 df) confirming a significant reduction in lifetime egg production in the treatment group compared with controls.

The mean total lifetime egg production (± C.L.) was 26.2 % ± 5.0 % lower in the treated group compared with controls.

4.4.4. Effects of permethrin on egg hatch rate

Figure 12. **Effect of adult permethrin exposure on eggs viability.**



$\chi^2_{MH} = 0.55$ (df=1) $p = 0.4598$ indicating there is no significant difference in the proportion of eggs hatching between treatment and control groups.

The mean (± C.L.) egg hatch rate was 89.59 % ± 7.1 % in the control group and 91.65 % ± 2.7 % in those exposed to sublethal doses with permethrin.

4.4.5. Effects of leg fracture on egg laying

Egg numbers per replicate are summarised in table 7. Following confirmation that the data were normally distributed (Anderson-Darling normality test p = 0.667), a one way analysis of variance confirmed there was no significant difference in the number of eggs laid by females with 0, 1 or 2 hind tarsae removed,

F = 0.05 (2/5 d.f.) p = 0.955 Confirming no significant difference between groups.

Table 7. **Effect of hind leg loss on egg numbers laid by *Anopheles gambiae*.**

Replicate	***Control***	***1 Leg removed***	***2 Legs removed***
1	2614	2841	2519
2	2781	2638	2742
3	2694	2553	2744
4	2316	2247	2370
5	2410	2298	2356
6	2443	2561	2608
Mean	2543	2523	2557

4.5. Discussion

Sublethal pyrethroid exposure was found not to directly affect the numbers of eggs laid per surviving female or their subsequent rate of hatching to viable larvae. This conclusion differs from those reached for a number of other insecticide classes with which fecundity and fertility effects are common. However, as an indirect effect, the reduced longevity as detailed in chapter 3 above, results in a fall in the mean egg numbers produced by a treated individual during its entire life time. In the case of Permethrin, the reduction in lifetime egg production was 13.98 % ± 6.2 % compared to untreated controls, with deltamethrin 23.6 % ± 5.1 %, and with lambda-cyhalothrin 26.2 % ± 5.0 %. This reduction occurs because exposed females die prematurely, allowing untreated control females to complete, on average, an additional two gonotrophic cycles. As a result, there is an effect on lifetime fecundity caused be the sublethal exposure to insecticides, which would presumably influence the next generation and give additional benefit in insecticidal control programmes aimed at reducing the vector or nuisance mosquito population. There was no significant change in egg viability following exposure to permethrin, with mean hatch rates of 89.59 % ± 7.1 % and 91.65 % ± 2.7 % in control and treatment groups, respectively.

Artificial leg fracture to simulate that associated with pyrethroid contact did not prove to be a significant hindrance to ovipositing females in the laboratory. Even those gravid individuals that had both hind legs removed still managed to lay as many viable eggs as controls. Whether this amputation would detrimentally effect subsequent host seeking, blood feeding and oviposition throughout the rest of the individual's life time was not, however, investigated.

CHAPTER 5 Effects on flight activity

5.1. Introduction

5.1.1. Normal mosquito activity

Biting insects have evolved feeding patterns which enable them to be at the right time and place to locate and feed on their preferred host. In anopheline mosquitoes used in the experiments described here, the normal host-seeking activity associated with feeding takes place predominantly at dusk, although in some species there is a smaller secondary peak of activity at dawn (Jones *et al.*, 1974). This bimodal circadian flight activity was observed by Jones & Gubbins (1978), in all female *Anopheles gambiae* except those having just blood fed or individuals less than 3 days post eclosion. The same group repeated the experiments in constant darkness and found that these peaks of host seeking activity remain constant even when conditions are changed to provide no dawn / dusk light stimuli. Under field conditions, there can be wide variation in circadian rhythms in different species, with some species reaching peak activity much later at night or exhibiting a unimodal, rather than bimodal, activity. Similarly, variation in activity may be a result of differing nutritional status (Clements 1999).

5.1.2. Measurement of flight activity

Early work on the activity of adult mosquitoes was carried out by direct observations, whether in the field (Haeger, 1955) or the laboratory (Kennedy, 1940). It was not until Jones (1964), first described a method for the automatic recording of mosquito activity that accurate and precise experimentation on multiple individuals could be considered. The device, which came to be known as an actograph, detected the sound generated during flight, and this sound was electronically amplified and bursts of flight activity were counted on a signal marker system. Since that time, many research groups have used similar devices based on this system (Rowland & Lindsay, 1986, Gomulski, 1988). With

the aid of such an electronic flight activity recorder, Hill (1989), reported a marked reduction in evening host seeking flights made by *An. albimanus* following sublethal contact with propoxur.

5.1.3. The influence of insecticides on activity

Many workers have reported that at low doses, and soon after initial contact with both DDT and pyrethroids, insects appear to become irritated or hyperactive (Hutzel, 1942 ; Kennedy, 1947 ; Ree & Loong, 1989). This initial excitation is a characteristic of the early stages of pyrethroid poisoning (Corbett *et al.*, 1984), and is considered by Matsungana (1991) to be a direct consequence of the repetitive discharges within the peripheral nervous system. During this stage it was observed by Khoo & Sutherland (1981) that the rapid sequence of strained leg flexing resulted in loss of legs due to fractures. In these experiments, using bioresmethrin on *Aedes aegypti,* it was found that the lower the final mortality after treatment the higher the incidence of leg fracture. Liu *et al.* (1986) also reported this phenomenon with three different pyrethroids in the same mosquito species. This irritancy may be thought of as having repellent-like effects which Haynes (1988) terms the 'sensory detection of the compound' rather than a neurotoxic event. Laboratory studies by Miller & Gibson (1994), used *An. gambiae* in wind tunnels with a video recorder to observe host-seeking females in contact with a permethrin-impregnated netting barrier. In this way, records were made of behaviour of individuals which were clearly irritated by the insecticide, making repeated short flights and continuing movements across the surface. From a field trial of permethrin impregnated bed nets by Jinjiang *et al.* (1988) it was concluded that one of the beneficial effects of such nets were that hungry female mosquitoes were repelled from the area. It is unclear as to the correct terminology to be used to explain such effects. True repellency is considered to be the sensory perception of a stimulus which results in a change in behaviour, whereas deterrency or irritancy depend more upon a neurotoxic action. Many field trials of

pyrethroids have recorded the occurrence of quite similar effects on insect activity, such as the observations by Hudsen & Esozed (1971) of a reduction in the number of *An. gambiae* that entered a room by around 50 % when using a pyrethrin coil, and of an even higher proportion (80-90 %) leaving without feeding. Taylor *et al.* (1981) reported a very similar effect against *An. gambiae* and *An. funestus* after spraying a room with permethrin in Kenya. In the control of Horn fly, *Haematobia irritans*, Quisneberry *et al.* (1984) concluded that part of the reason for a decline in control efficacy with pyrethroids was the fact that partially resistant flies took flight more frequently from treated hosts. Such increase in activity is a characteristic of low dose or short contact time with such compounds but, at higher doses or after a longer period post-contact, the hyper-excitatory phase is often replaced by a fall in activity, or paralysis, which is due to the eventual blocking of the neurons following prolonged repetitive firing. Boiteau *et al.* (1985) found that aphids surviving aldicarb treatment had a much lower flight activity than controls. Similarly, a decrease in locomotory activity was recorded by Armstrong & Bonner (1985) in *Drosophila melanogaster* exposed to permethrin. The effect of sublethal doses of pyrethroids on honeybees (*Apis mellifera)* is an interesting situation studied by several groups. Rieth & Levin (1988), found permethrin and cypermethrin to be contact repellents which resulted in transitory inhibition of activity reversible within 24 hours. Such irritancy, resulting in no lasting damage to honeybees, is clearly a benefit environmentally over many other classes of crop pesticides which often kill both harmful and beneficial species indiscriminately. Later work by Mamood & Waller (1990) found permethrin treated bees not only showed signs of abnormal activity but were unable to be trained by the classic odour-mediated learning response. This learning impairment lasted for four days post treatment, but those bees which had been trained prior to permethrin exposure did not lose this ability to respond to odours. Laboratory trials by Evans (1993), using *An. gambiae* in test cages, showed excito-repellency using a range of insecticides including DDT and lambda-cyhalothrin. The experiments indicate the

relatively short contact time, larger number of take-offs and increased time spent in flight when using the pyrethroid compared to carbamates or controls. However, as the contact time required for mortality is very short with pyrethroids, and the fact that much of this study used doses substantially lower than recommended field application rates, the conclusions which can be drawn from the study are rather limited. Similar laboratory tests on field caught *An. farauti* and *An. maculatus* held in permethrin treated or control chambers by Ree & Loong (1989) present similar increases in irritability, although to a much lower degree in the latter species.

5.1.4. Other factors which modify activity

As well as insecticide contact, normal flight activity can be influenced by a range of parasites and infections. Berry *et al.* (1986), for instance, reported that loss of flight activity by *Ae. aegypti* was inversely correlated to levels of infection with *Brugia pahangi*, no doubt as a direct result of parasite invasion and subsequent destruction of the flight muscles. These findings were reinforced by Rowland & Lindsay (1986) who used an actograph to record flights in the same parasite / host combination, and verified further by one of the authors, Lindsay (1986) having previously reported the migration of larvae within tissues of this mosquito species. No similar physical tissue damage results from viral infections and this would account for Berry *et al.* (1987) failing to detect any abnormalities in flight activity of another *Aedes* species infected with trivittatus virus. Kurihara *et al.* (1991) found that *An. stephensi* infected with rodent malaria (*Plasmodium yoelii nigeriensis)* were less likely to fly from one cage to another through a wide mesh net. Shortly afterwards, this same group reported that this reduced activity was dependent upon the number of *Plasmodium yoelii nigeriensis* oocysts per gut, and hence the level of infection (Kurihara & Kikuchi, 1991) Schiefer *et al.* (1977) found a similar reduced activity in *An. stephensi* infected with *Plasmodium cynomolgi*, possibly as a result of direct competition between host and parasite for reserves of a metabolic substrate. This

depletion of available flight reserves by trypanosome parasites in tsetse flies was estimated to be as high as 15 % by Bursell (1981).

5.1.5. Aims of this chapter

Adult mosquito activity is clearly an important factor which determines disease transmission. Females need to fly periodically throughout their life to reach a host and again to oviposit. Similarly, their activity determines whether, and for how long, they come into contact with insecticide treated surfaces, and thus the efficacy of control programmes such as residual spraying and impregnated bed nets. This study aims to explore the immediate and delayed effects of pyrethroid contact on the activity of female *An. gambiae*. In particular, it aims to investigate abnormal activity during contact with a treated surface, as well as any longer-term effects on evening flight activity associated with host location.

5.2. Materials

5.2.1. Mosquitoes

ZANU	*Anopheles gambiae*

5.2.2. Insecticides

Permethrin	0.25 % on paper
Deltamethrin	0.025% on paper
Lambda-cyhalothrin	0.025% on paper

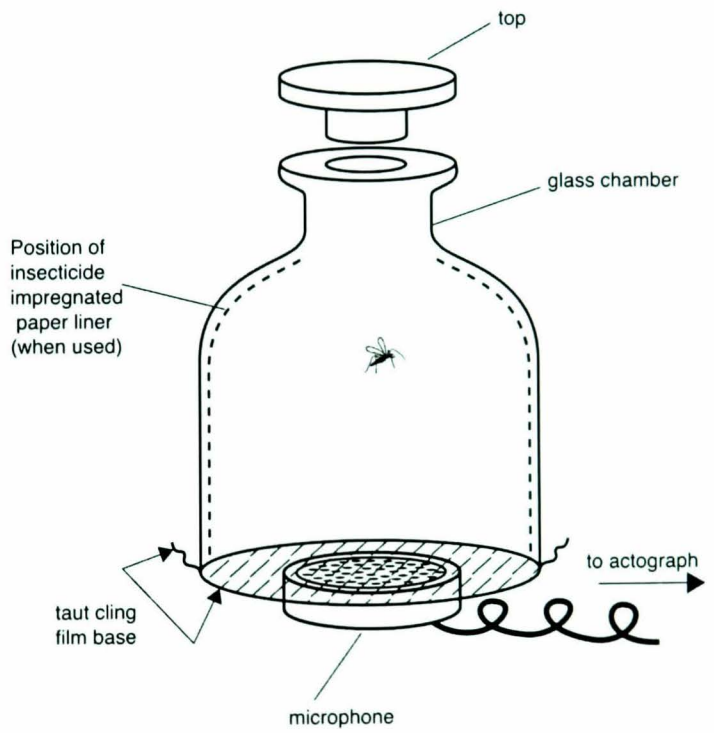
5.3. Methods

5.3.1. Recording flight activity

An acoustic actograph was constructed based on that designed by Jones et al. (1964), and subsequently used by many others (Jones *et al.*, 1974; Jones & Gubbins, 1978; Rowland & Lindsay, 1986; Gomulski, 1988). Individual female mosquitoes were isolated in a glass

container which rests over a microphone in an insulated, sound-proof box as shown in figure 13. Several such containers, each with its own microphone, are connected to an electronic device which amplifies and records the sounds of the insects in flight. By both rearing and testing the insects in the same regulated light/dark cycle it was possible to record the evening host-seeking flight activity associated with anopheline females. The actograph was set to record the period of 15 minutes before, and 2 hours after, lights out. The activity in each chamber was recorded giving the number and duration of flights by each female per minute. This procedure was repeated over several days using fresh females in each case and with chambers allocated at random.

Figure 13. **Diagram of an actograph chamber.**



Activity data for each chamber was recorded onto a paper printout by a chart recorder and converted into a score of 0 - 10 based on the proportion of time spent in flight per minute. A score of 0 would indicate no flight activity took place, 10 would indicate either 10 short flights were made or a single flight lasting the entire one minute period.

5.3.2. Contact behavior

Glass actograph chambers were lined with Whatman's No. 1 filter paper impregnated with a pyrethroid or silicone oil control covering all surfaces and held in place with a thin metal band. Three-day-old, mated "ZANU" females were then placed at random individually into the chambers during mid-morning when the females would normally be mostly inactive. The actograph was switched on after 5 minutes and activity was recorded for a period of 30 minutes, after which the mosquitoes were observed for knockdown or mortality. This was repeated 6 times over several days with batches of 10 fresh insects each time, and with decontaminated glass chambers on each occasion.

5.3.3. Effect of permethrin on subsequent flight activity

Two random groups of "ZANU" females were collected after allowing them to mate freely for 3 days, but without having received a blood meal. One group was given a sublethal (15 minute) dose of pyrethroid and the other contact with a silicone oil control, then both were allowed 24 hours to recover and the survivors were used in the subsequent experiments. Eight females, four treated and four controls were placed individually in the glass actograph chambers 2 hours prior to lights out. Any individual recording no flight at all at the end of a recording period was checked to ensure it was still alive before data were used for further analysis. Eight replicates of 8 females were conducted.

5.4. Results

5.4.1. Effects on contact behaviour

Figures 14 – 16 clearly show marked increases in flight activity of pyrethroid exposed females at a time when normal control females are almost entirely inactive.

Figure 14. **Effect of permethrin contact on spontaneous flight activity of female *Anopheles gambiae* during a period of normal inactivity (n= 6 replicates of 10).**

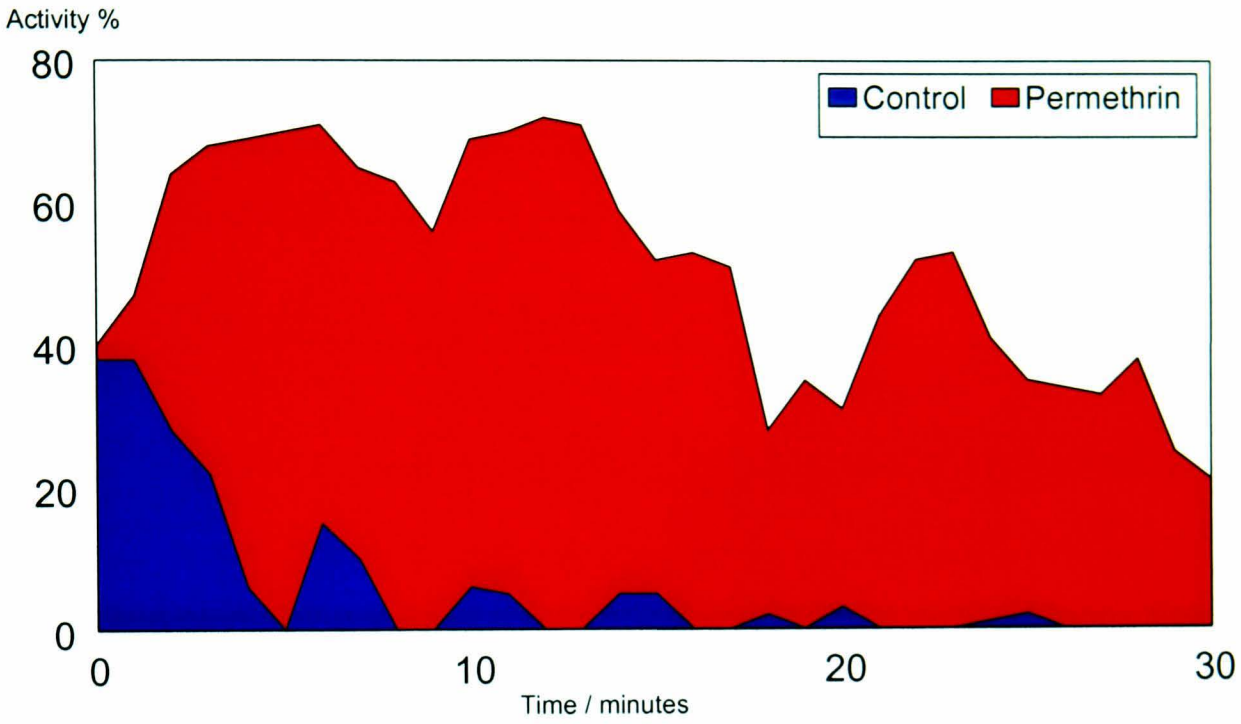


Figure 15. **Effect of deltamethrin contact on spontaneous flight activity of female *Anopheles gambiae* during a period of normal inactivity (n = 6 replicates of 10).**

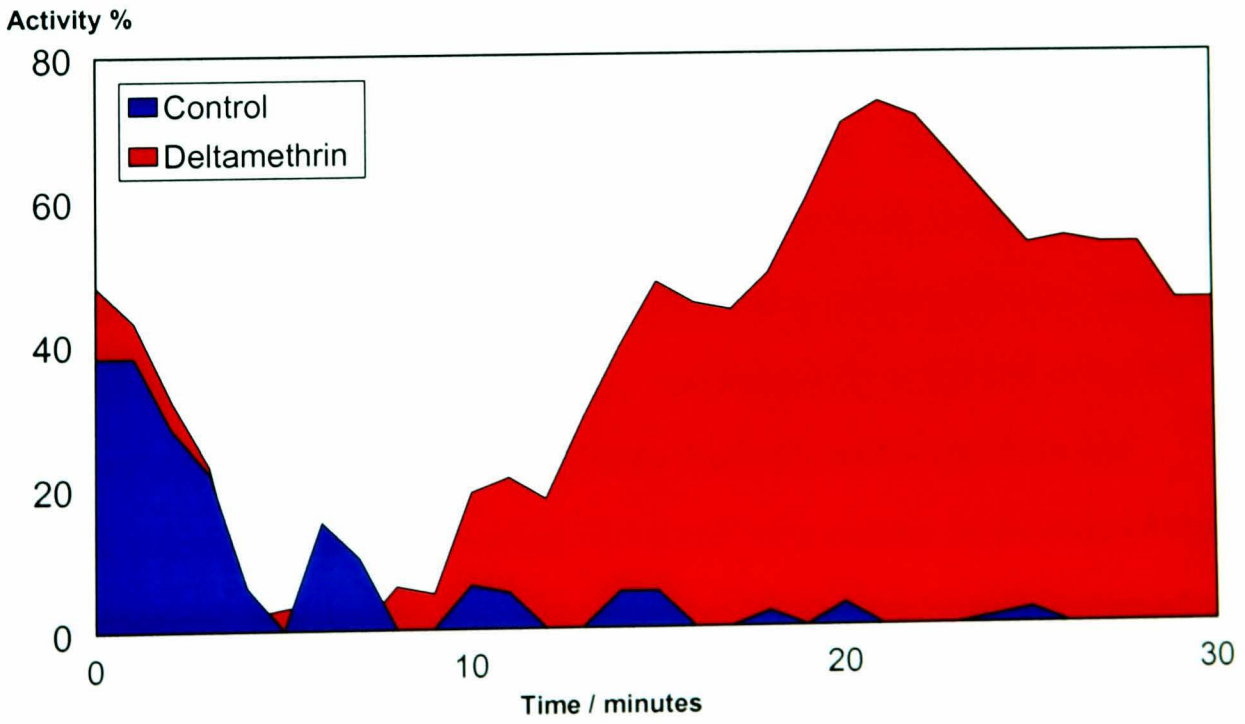
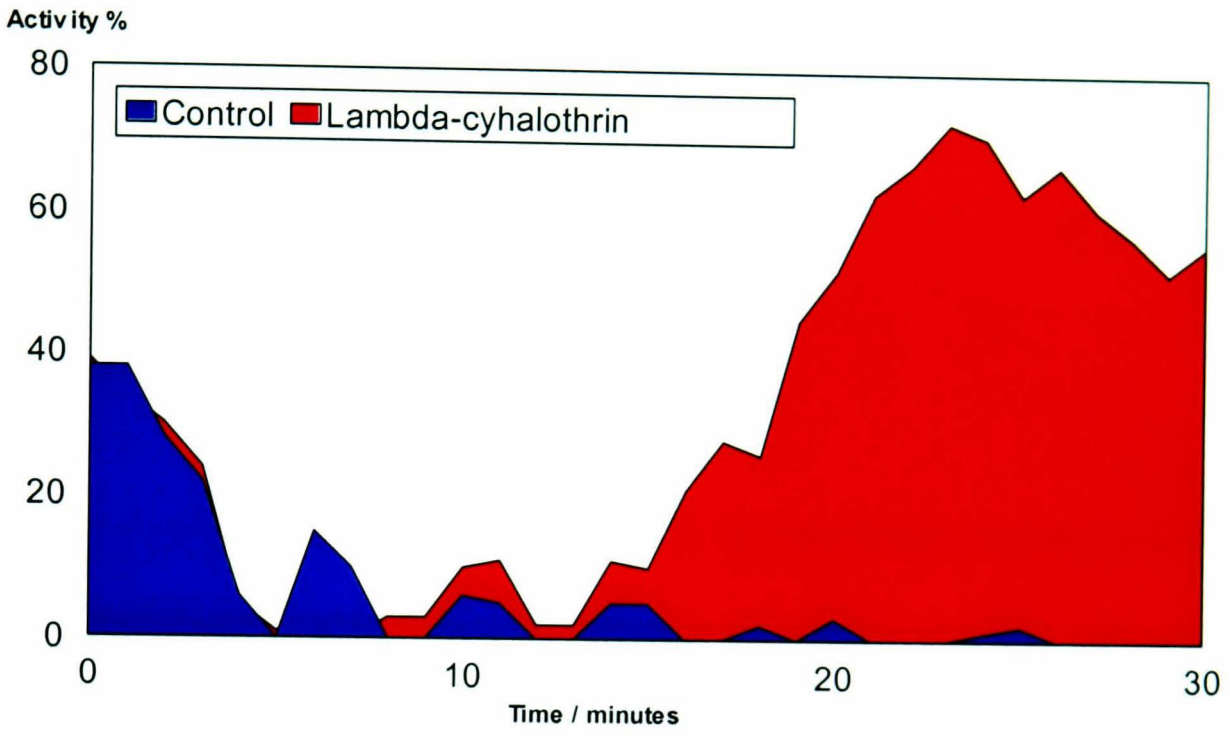


Figure 16. **Effect of lambda-cyhalothrin contact on spontaneous flight activity of female *Anopheles gambiae* during a period of normal inactivity (n = 6 replicates of 10).**



Every individual, exposed to any of the 3 pyrethroids used, demonstrated a spontaneous activity which continued until recording stopped at 30 minutes or the individual was knocked down. It can also be seen that the activity began earlier in those mosquitoes in contact with permethrin than in the case of either lambda-cyhalothrin or deltamethrin, implying that the former compound is more irritating to the insects. It is likely that the initial burst of activity, from time zero to around 5 minutes, is a direct result of the females being disturbed immediately after being placed in the chambers, as the peak was observed in all treatments and mirrored in the control groups. On inspection at the end of the 30 minute recording period it was evident that many individuals in the deltamethrin and lambda-cyhalothrin chambers had been knocked down and were resting on the base of the chambers. As is common with pyrethroids, the majority of these individuals continued to rapidly twitch legs and flap wings in a convulsive manner. It is therefore likely that much

of the dramatic increase in activity seen towards the end of the recording periods (15 minutes +) is not true flight activity but a result of knockdown, particularly as it was less pronounced in the permethrin treated group where fewer individuals exhibited this phenomenon.

5.4.2. Effects on subsequent flight activity

As shown in figures 17 – 19, all three pyrethroids caused a reduction in evening flight activity compared to control females.

Figure 17. **Effect of prior exposure to permethrin on evening female host-seeking flight activity in *Anopheles gambiae* (n = 8 replicates of 8).**

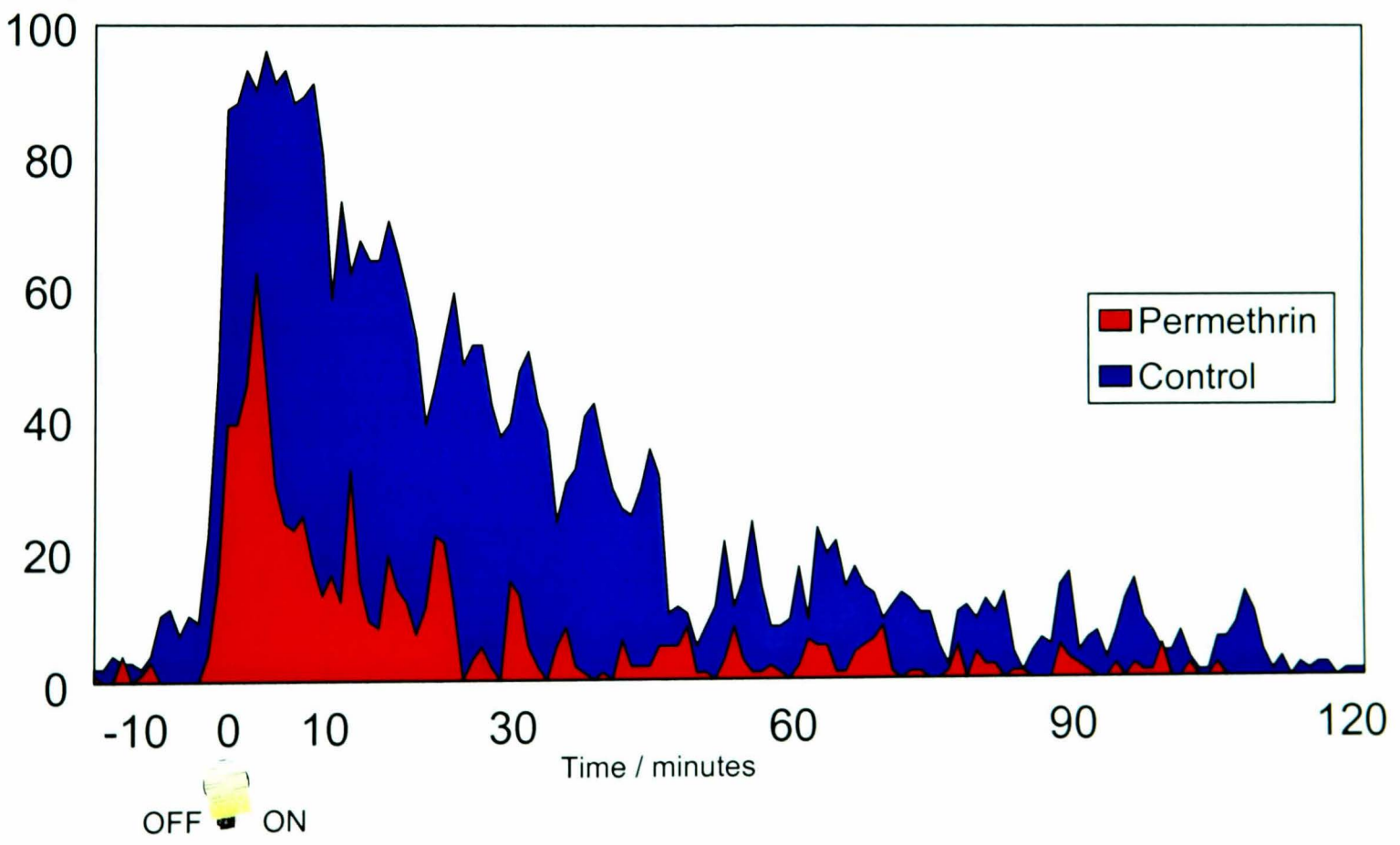


Figure 18. **Effect of prior exposure to deltamethrin on evening female host-seeking flight activity in *Anopheles gambiae* (n = 8 replicates of 8).**

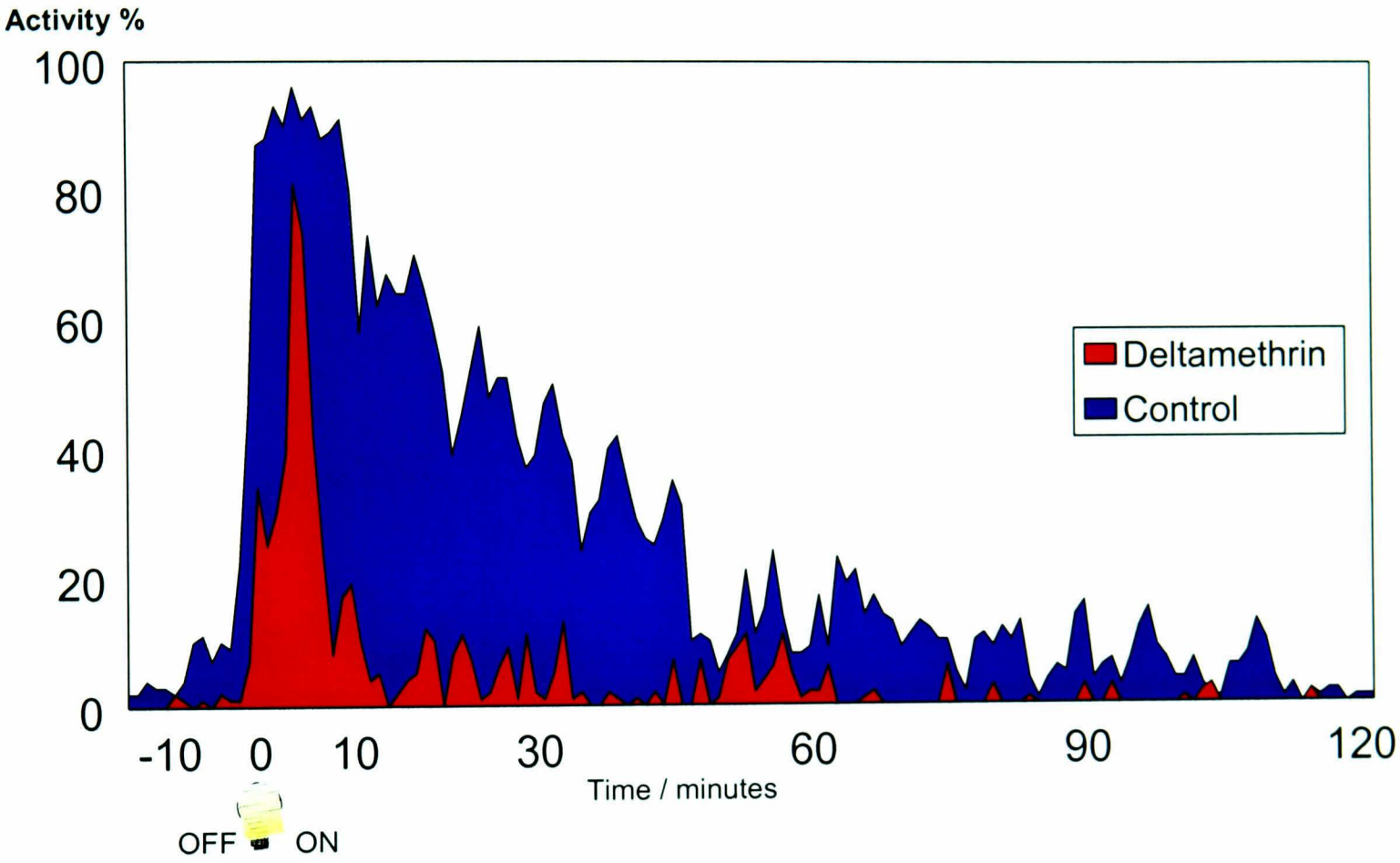
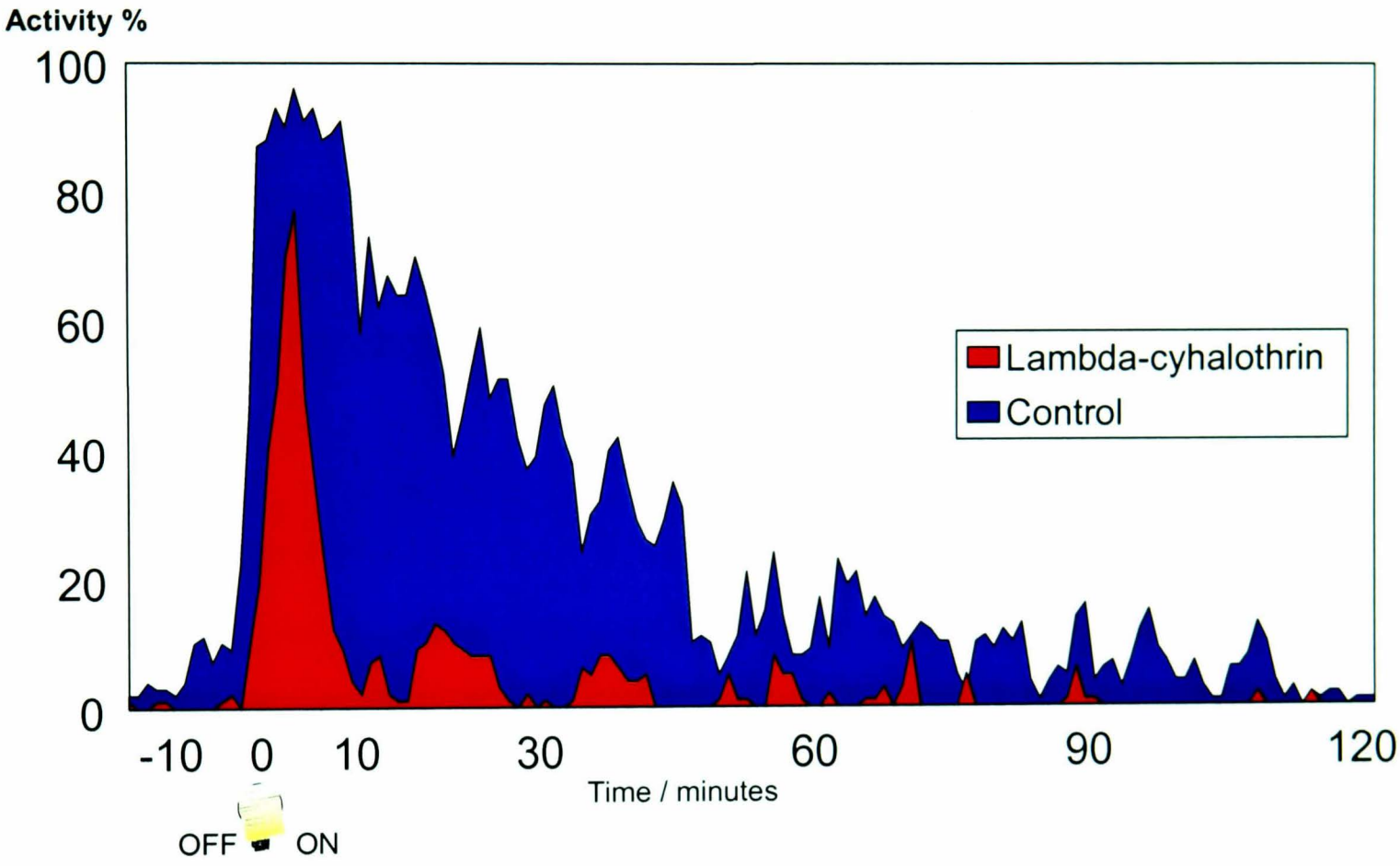


Figure 19. **Effect of prior exposure to lambda-cyhalothrin on evening female host-seeking flight activity in *Anopheles gambiae* (n = 8 replicates of 8).**



All three pyrethroids appear to almost eliminate this evening behaviour on the evening after sublethal contact, with just a few short flights being undertaken in the early period post lights out.

5.5. Discussion

Observation of the female host seeking behaviour, most notably the early evening flight activity characteristic of *An. gambiae*, following sublethal contact with three pyrethroids investigated reveals a common trend. In all cases there was a marked reduction in

spontaneous flight activity in the laboratory at a time when control females were at their most active. Lambda-cyhalothrin and deltamethrin suppressed the activity almost completely beyond the immediate "light off" point, and permethrin was found to limit flights to a few short bursts early in the expected period. The reduction in activity may be attributable to exhaustion as a result of abnormally high activity following contact with the insecticides, or perhaps a more direct effect on flight muscles or their nervous control. This action of pyrethroids on normal flight activity could be important when considered in terms of what effects may be detected in the field. Mosquitoes having survived contact with the insecticide would be less likely to disperse, seek a host or search for breeding sites. All of these features could be expected to aid a vector-borne disease control programme.

Normally inactive females, when placed in an actograph chamber lined with a control paper, were found to exhibit little or no flight activity once the initial disturbance of handling was over. In chambers lined with permethrin, however, there was a marked and sustained activity for a period of up to 30 minutes or until knockdown. Clearly contact with the insecticide is either detected by the mosquito which then attempts to leave the treated surface or, alternatively, sublethal poisoning results in involuntary motor neuron activity causing detectable movement in the chamber. In the case of both deltamethrin and lambda-cyhalothrin, it appears that knockdown occurs so rapidly that detection of the compounds by the insects does not take place and little activity is recorded prior to knockdown and death. This fact could be very important in their use in vector control as, in such cases, it is undesirable for the individual to be repelled from a treated surface prior to acquiring a lethal dose, as it may simply fly to another, unprotected host elsewhere. Some failures in DDT spraying have been put down to the mosquito population altering their normal resting behaviour to spend less time on treated surfaces thus avoiding contact. Possible reasons for this include individual mosquitoes becoming

irritated during contact with DDT sprayed walls, or a genetic change in the population resulting in switching from endophily to exophily. Such behavioural resistance has been reported by Suwonkerd *et al.* (1990) in *An. dirus* in Thailand and by Li *et al.* (1983) in *An. minimus* on Hainan island, China. However, one would not expect a similar problem with these very fast acting pyrethroids which are unlikely to be detected before knockdown and death, as demonstrated by Miller and Gibson (1994).

CHAPTER 6 Effects on feeding behaviour

6.1. Introduction

As has already been demonstrated, the effects of sublethal pyrethroid exposure are widespread and examples of abnormal physiology and behaviour can be found in many areas. One behavioural trait of particular relevance in the transmission of vector-borne diseases is that of host location and blood feeding.

6.1.1. Host location

It is well known that many stimuli and interactive behavioural events combine to initiate and maintain the seemingly simple act of locating and homing in on a potential host. Edman (1991) suggested splitting the overall activity of host location and biting into 3 distinct phases, these being i) distant orientation, ii) near orientation and landing and iii) blood feeding. This view of dividing host seeking into long and short distance attraction is shared by Takken (1991) who reviews the early work on olfactory chemosensillae. Although segmenting what at first glance seems to be one behavioural act may appear to be a gross oversimplification, particularly in the case of anopheline mosquitoes, it does appear to have some justification. An anopheline female may approach and enter a house during the early evening but then rest indoors for a considerable period before attempting to feed later that night. Such behaviour does not constitute a continuum of activity so the assumption of several independent stages of host seeking behaviour seems to be valid. Similarly, females brought into direct contact with a blood source will often feed yet have not undergone host seeking prior to the act of feeding. Despite this simplification of such behavioural events it is important to remember that, although these phases may be under separate physiological control, they remain interrelated to a high degree, for example, females which are not ready to blood feed cannot be induced to search for a host.

6.1.2. Distant orientation

Insects such as the tsetse fly, *Glossina,* move upwind following a graduated odour plume (anemotaxis), either directly as suggested by Brady *et al.* (1989), or indirectly as substances in the plume initiate a series of short bursts of upwind flight, as described by Payne *et al.* (1986). Some form of optomotor response using visual clues is generally believed to aid in this flight (Sutcliffe, 1987) although Gillett (1979) has suggested information on wind-shear gained from periodic dipping close to the ground could be used to gain the same information by mosquitoes flying on very dark nights. The distance of orientation flights depends greatly on the species. In the case of *An. gambiae* it has been recorded occurring at least 40 m from paired hosts of a calf and human bait by Edman (1991), and could reasonably be expected to extend further to larger host concentrations such as villages. Host associated cues include visual detection although, in night flying vectors such as anophelines, the importance of these may well be limited. Gillies & Wilkes (1982) speculated that mosquitoes orientated from a distance towards dwellings using sight, as did Bidlingmayer & Hem (1980) observing several night flying species of mosquito arriving at large objects from a distance of 19 m. Chemical attractants from hosts to female mosquitoes have been well documented. Carbon dioxide is a well known stimulus although at high doses it also acts as a repellent, but even at optimal doses it is still far from a full explanation of host attraction as demonstrated when Gillies (1980) compared it against a live host. When CO_2 is augmented by adding other animal odours greater attraction is observed, as Kline *et al.* (1990) demonstrated with lactic acid and / or octenol. Different odours emanating from various regions of a host body may act as stimuli for different mosquito species, which may account for the fact that they may bite on distinct parts of the body. De Jong & Knols (1995) studied the landing sites of *An. gambiae s.s.* and *An. atroparvus* on a human body and found that these species tend to select the foot and head regions respectively. Furthermore, by thoroughly washing the feet and ankles with anti-bacterial soap it was possible to divert *An. gambiae* to other parts

of the body suggesting that the attractant was a kairomone and not a thermal or visual cue. Interestingly, these results were thought to explain the differing host preferences of the two species, *An. atroparvus* being an opportunistic feeder responding to CO_2 released by all animals, whilst the highly anthropophilic *An. gambiae* requires a more specific chemical cue from human feet. Indeed, when this explanation was investigated in the field by Dekker & Takken, (1998), it was verified by trapping many zoophilic *An. quadriannulatus,* but few man biting *An. arabiensis,* using high CO_2 emissions but the reverse with low CO_2. Much work has been conducted to isolate possible kairomones over the years. Eiras & Jepson (1994) developed an olfactory bioasssay utilising various host odours in a convection current. They reported that human sweat extracts elicited a much higher response in *Ae. aegypt* females than lactic acid and water vapour alone. The best attractant of all was, predictably, a human hand, which indicates that despite our increased understanding of host attraction it is clear that several other important kairomones have yet to be isolated. Such substances, if and when detected, may play an important role in explaining some of the well documented host specific species of mosquitoes, like those described by Magesa *et al.* (1991) in Tanzania where most of the *An. gambiae* studied by ELISA had fed on humans, whereas the majority of *An. funestus s.l.* on cows. Similar African *Anopheles* data reported by Gillies (1972) suggests that of 72 species studied sufficiently, 7 habitually feed on man, 59 on any large animal and 6 on small mammals. Such host preference is clearly not, however, a determinant of the distribution of mosquito species. For example, reports of *An. arabiensis* being present in an area of South Africa devoid of human habitation was clearly a surprise to Braack *et al.* (1994), following earlier work by Sharp & Le Sueur (1991) which had described the species as having an overwhelming preference for man in that country.

Although such host preference is apparently under genetic control, Edman (1989) believes it to be regulated at the preliminary distant orientation level. If therefore, this

behaviour could be disrupted, a change in host could be achieved, and, if this was from man to other animals, it could mean a significant benefit in terms of malaria control; indeed this was one effect that Magesa *et al.* (1991) were hoping to observe during bed net trials in Tanzania.

6.1.3. Near orientation and landing

CO_2 and other host odours are important at a distance and they may still elicit a response at close range. However, there are other cues which play an additional role during the final approach and landing. Thermoreceptors have been identified on mosquito antennae by Bowen (1991) which are sensitive to very small changes in temperature, possibly even being solely used to guide approach to a host following a temperature gradient in the absence of other cues. Wright & Kellogg (1964) discovered that even in still air, like that within a dwelling, the localised emanation of warm, moist convection currents from a host can carry chemicals from the skin and breath to enable detection by mosquitoes over short distances. Edman (1991) presumes, although seemingly without providing any evidence, that different stimuli are used to determine final landing on the host. Visual recognition of the host surface is very likely to play a substantial role in day biting insects, but this may be less important in night biters such as anophelines. For example, when comparing the landing responses of *Aedes* and *Anopheles*, Klein *et al.* (1990) found the night flying anophelines to be more temperature dependent than daytime feeding *Aedes*.

6.1.4. Biting and blood feeding

Having successfully found and alighted on a host the mosquito initiates a probe response, and must locate a venule or arteriole. In *An. stephensi* studied by Li & Rossignol (1992) the first probe is far longer than subsequent attempts, and the probability of locating blood, initially high, falls with time. Saliva is secreted continually during the feed from probing to withdrawal of the mouthparts, as demonstrated by Golenda *et al.* (1995), who recorded

a proportional decrease in total salivary gland protein with feeding duration. It has been shown that mosquitoes which have been surgically deprived of saliva have great difficulty in locating blood (Riberio *et al.*, 1984). This group suggest that without the apyrase in saliva to hydrolyse ATP and ADP, there is increased platelet aggregation, which reduces haematoma formation during probing, thus hindering blood location and uptake. The success of feeding depends upon many factors, not all of which are in the tarsi of the insect. Host defence behaviour, such as grooming or swishing of tails, can increase the number of interrupted feeds and subsequently reduce the numbers of individuals feeding to repletion (Davies 1990). Choice of host, length of the feeding process and pain of the bite can all influence feeding success, as can the persistence of the hungry female. Walker & Edman (1985), working with *Ae. triseriatus* in the laboratory, found a finite willingness to return to a hand having been repeatedly dislodged after landing to feed. Interestingly this persistence proved to be dependent to some extent upon the nutritional status of the individuals, those sugar fed with high energy levels trying for longer than starved individuals. The earlier a blood meal is taken by an individual after emergence the greater the energetic advantage, as demonstrated by Naksathit *et al.* (1999), who found a feed taken by *Ae. aegypti* on or before day 2 maintained a significantly higher lipid level throughout later life. In addition, the size and nutritional status of a female will influence feeding rates. Takken *et al.* (1998), for example, found that small female *An. gambiae* frequently omitted oviposition between feeds and sought hosts more frequently than their larger counterparts.

6.1.5. Feeding behaviour and disease transmission

Host feeding behaviour will clearly have a direct effect on disease transmission where parasites are introduced to the host during the feeding process. The host preference of a species capable of carrying a disease will dictate whether or not it is a potential vector for human transmission, whilst other factors, such as distribution, feeding frequency and

population size will determine transmission rates. The host preferences of many anopheline species have been reported (Sharp & le Sueur, 1991, Rodriguez *et al.*, 1992, Muirhead-Thomson, 1982). Those species found to be infrequent or opportunistic human feeders may be successfully diverted from man onto other animals if they are introduced close to human habitation. Diversion due to an insecticide was clearly demonstrated by Sharp & le Sueur (1991), where the proportion of human : bovine blood feeds by *An. arabiensis* fell from 90.4 : 9.6 to 31.2 : 68.8 following DDT spraying. Strongly anthropophilic species will clearly be harder to deter in this way to reduce transmission. The gonotrophic cycle of the vector species will influence the frequency with which an infected individual will attempt to locate and feed on a host. It is usually, but not always, the case that, once fully fed, a mosquito will not seek another host until an egg batch has been laid. After a successful blood meal, inhibition of host seeking is mediated by two mechanisms. Firstly, the triggering of stretch receptors in the anterior abdomen (Klowden, 1983), and secondly, a haemolymph-borne factor released by the fat body in response to a humoral signal from the vitellogenic ovary (Klowden, 1987). The second of these mechanisms acts by reducing the sensitivity of the peripheral lactic acid receptors which are critical to location of a host (Bowen *et al.*, 1988). This group found that the lactic acid dose-response curve of grooved peg sensillae in diapausing *Culex pipiens* shifted to the right, suggesting that the receptors became less sensitive during periods when insects were not responsive to host-seeking.

In most cases, those research groups working in the field assume that mosquitoes only seek a new blood meal after having oviposited the previous egg batch (Lines *et al.*, 1990, Rodriguez *et al.*, 1992). However, Scott *et al.* (1993), using a histological technique, detected that up to 50 % of wild caught Puerto Rican *Ae. aegypti* had taken blood meals on two consecutive days, and that the phenomenon was not restricted to the first gonotrophic cycle. Using similar techniques, Wekesa *et al.* (1997) later confirmed

that this was the case in 13 % and 10 %, respectively, of wild caught *An. freeborni* and *Cx. tarsalis*. Field experiments by Anderson & Brust (1995), on wild *Cx. tarsalis* attracted to traps baited with two quail, with their blood differentially labeled with rubidium or caesium, confirmed this. Klowden & Briegel (1994), conducted laboratory trials on two aedine and three anopheline species in an olfactometer. In their tests, all three anopheline species exhibited varying degrees of attraction to hosts despite currently maturing an egg batch. In the laboratory too this effect has been observed. When *Ae. aegypti* were offered blood meals at 6, 12 and 24 hours post-feed by Canyon *et al.* (1999) there was a small proportion of females which took additional feeds within any 24 hour period. The fact that some mosquitoes do continue to exhibit host seeking behaviour may be due to the fact that two separate inhibitory factors are in operation. Enough blood to begin vitellogenesis may not be enough to trigger distension-induced inhibition, the result of which may be a further period of stimulation by host cues.

The act of feeding itself can be modified in some hosts by the disease it is carrying. Examples of this are seen frequently in arbovirology. Grimstad *et al.* (1980), reported that *Ae. triseriatus* infected with La Crosse virus probed longer and engorged less than uninfected females. As probing is sufficient to transmit the virus, it likely that, as a result of this manipulation of the mosquito's behaviour by the virus, an infected individual will be a more efficient vector, as a larger number of hosts could be visited in an attempt to complete a full feed. Conversely, Mellink (1982), found *Ae. aegypti* infected with Venezuelan Equine Encephalomyelitis virus which fed for a prolonged period resulted in lower levels of infection in a host. The authors believe that this was due to the virus particles being re-imbibed by the mosquito during uptake of blood, a circumstance also postulated by Hurlbut (1966), investigating transmission of St. Louis Encephalitis virus by *Cx. pipiens pipiens*. Fleas infective with plague are perhaps the classic example of feeding modification to benefit the parasite, by blocking the gut they ensure future feeding

attempts result in regurgitation of blood plus bacteria into a new prospective host thus heightening the chances of the bacteria being passed through the population. *Ae. aegypti* infected with *Lambornella clarki* were found by Egerter & Anderson (1989) to be inhibited in seeking a host and those that did so took approximately twice as long to probe and engorge compared to uninfected controls. A similar effect may be present in mosquito / malaria relationships, for although the presence of oocysts was found by Kurihara *et al.* (1992) to reduce flight activity in *An. stephensi* this trend reversed when oocysts burst and infective sporozoites developed. In this connection, Edman (1991) reports studies in Kenya suggesting that malaria infected females are more likely to land on hosts, probe longer and do so more frequently.

6.1.6. Effects of sublethal doses on feeding behaviour

Feeding behaviour was observed in the tsetse fly, *Glossina morsitans*, 48 hours post treatment with dieldrin, endosulfan or permethrin by Kwan & Gatehouse (1978) and reduced bloodmeal size was recorded. Liu *et al.* (1986) also found significant reduction in blood engorgement, using a range of doses of three different pyrethroids in the mosquito *Ae. aegypti*, but the authors could offer no explanation of the mechanism for this depressive effect. One possible cause for such changes in behaviour was investigated by Chadd & Brady (1982), who recorded the effects of sublethal doses of permethrin on tsetse flies and blowflies. Although DDT was seen to cause a temporary increase in the probing responsiveness, permethrin resulted in a prolonged and marked reduction in this primary feeding behaviour. In the early stages of poisoning with permethrin, Fryauff *et al.* (1994), reported an increase in biting activity in the tick *Hyalomma dromedarii*. Ticks exposed to materials impregnated with permethrin had attached to hosts in a higher proportion and more rapidly than controls. The authors postulate that this action was induced by premature or excessive release of a neurosecretory substance which elicits pre-feeding attachment. Alford (1991) noticed a reduction in food consumption by the spruce

budworm, *Choristoneura fumiterana*, when low levels of fenitrothion were included in the diet and that, following exposure to carbaryl or aminocarb, significantly less food was consumed than by untreated controls. Permethrin has been found to inhibit feeding and subsequent fecundity in the two-spotted spider mite, *Tetranychus urticae*, when applied at sublethal doses by Hall & Thacker (1993) to their bean leaf diet. The same compound was seen to inhibit feeding of *Drosophila melanogaster* adults by Armstrong & Eonner (1985), whilst increasing preening activity, perhaps in an attempt to remove sensory surface contaminants. The biting midge, *Culicoides variipennis*, when feeding on permethrin treated goat hair over membrane feeders, achieved only partial engorgement during investigations by Mullens (1993). An interesting phenomenon was reported by Leprince *et al.* (1992) whereby tabanid horse flies were found to take the same volume of blood as controls when biting lambda-cyhalothrin ear-tagged bullocks, but in a much shorter period of feeding. Such an effect seems to be a feature of this pest species as Foil *et al.* (1990) found a similar accelerated feeding rate on cattle sprayed with fenvalerate.

Insecticides are not the only chemicals which affect feeding responses. Azambuja & Garcia (1991) report that the hormone disrupting substances, precocenes, also resulted in reduced feeding when added to the blood source of *Rhodnius prolixus*. An insect growth regulator, hexaflumuron, when exposed at sublethal doses to the larvae of *Cx. quinquefasciatus*, *Ae. aegypti* and *An. stephensi* resulted in emergent adults exhibiting suppressed blood feeding rates and, overall, fewer females took a blood meal. The authors suggest that this was due to physical abnormalities in the mouthparts but also acknowledge the possibility of disruption of normal behaviour. Another compound known to inhibit feeding, azadirachtin from the neem tree, *Azadirachta indica*, was found to cause weight loss and increase mortality during moulting of Lepidopterous larvae of several species (Simmonds & Blaney, 1983). The authors studied neuro-physiological responses in maxilla sensillae and concluded that the compound stimulated specific

deterrent neurons in some species and triggered firing of neurons controlling phagostimulants in others. Other anti-feedants have been reported such as essential oils derived from the plant sweetflag, which was found by Koul & Isman (1990) to inhibit feeding and growth of caterpillars feeding on cabbages. Ismail & Matsumura (1991) induced intense anorexia in *Periplaneta americana*, the American cockroach, with very low doses of a formamidine pesticide, which was attributed to excessive metabolism in thoracic muscles resulting in the abnormal conversion of trehalose to glucose.

6.1.7. Aims of this chapter

This study aims to build on the findings in chapter 5 by investigating in more detail the very specific and important types of activity associated with host location and feeding behaviour. The ability to locate, approach, land and ultimately feed upon a potential host is not only crucial to the individual's survival, but fundamental to its potential to transmit disease. Flight activity and host seeking behaviour was studied under more realistic experimental conditions in a free-flying chamber with a human host. Effects of exposure to pyrethroids on host seeking activity and post-landing probing response were observed at various time points following contact. Findings are discussed in relation to possible effects on disease transmission and vector control programmes which use these compounds.

6.2. Materials

6.2.1. Mosquitoes

ZANDS *Anopheles gambiae*

6.2.2. Insecticides

Permethrin	0.25 % papers
Deltamethrin	0.025 % papers
Lambda-cyhalothrin	0.025 % papers

6.3. Methods

6.3.1. Host seeking behaviour

Adult "ZANDS" were reared in the standard way and left for 72 hours, after which time it was assumed they would have mated and the females would be ready to take a blood meal. Two random groups were divided and exposed to permethrin or an oil control and then left for a specific recovery period of either 24, 48 or 72 hours. After the specified recovery periods, 20 hungry females were selected from the treated insects and these were released into a test area and allowed to fly freely to feed on a human host. Only females attempting to probe on a hand placed close to the side of the holding cage were used, ensuring that all individuals were hungry. The sealed test room was 13 cubic meters, and maintained at 24°C ± 1°C, with white walls, ceiling and floor to allow easy visibility of mosquitoes during and after testing. The human host had bare feet and lower legs and, following release of the females, counted and collected those landing on the skin to feed. After a period of 12 minutes counting stopped and all the mosquitoes were removed from the room. A comparison test was then carried out using the females of the control group; any differences in numbers of bites would be a reflection of the effects of sublethal doses of permethrin on the short range host seeking response. Six replicates of 20 females were conducted with both the treatment and control groups.

6.3.2. Probe response

Standard mosquitoes were allowed to mate for four days, then females were chosen at random for exposure to permethrin or an oil control and their subsequent probing response on a human arm was observed for up to 5 minutes. To ensure that only this single response was measured, the females were relieved of the need for host seeking behaviour by placing them directly onto the skin by means of a 'tether' consisting of a single filament of dental floss attached to the thorax by wax. The single filament was separated from the

dental floss and, with care, lengths of about 30 cm, much finer than a single hair could be attached to the mosquito and the other end to a reference tag. The act of inserting the proboscis through the skin and subsequent feeding was observed, with the majority of control females completing a feed within 5 minutes following a single probing attempt. Those females taking more than two attempts to probe, i.e. inserting and withdrawing the proboscis on three or more occasions prior to, or part way through, feeding, were recorded as cases of abnormal 'multi-probes'. Females were lightly anaesthetised with chloroform to knock them down for just long enough to affix the tether, around 30 seconds. When fully recovered, they were given a specific period to recover from insecticide or silicone oil exposure before being offered a human arm. The recovery times post exposure were 2 minutes for which the tether was applied before treatment, and 2 hours or 24 hours, where the tether was applied during the treatment recovery period. Batches of between 8 and 10 females were used in both control and treatment groups and a total of 8 replicates conducted.

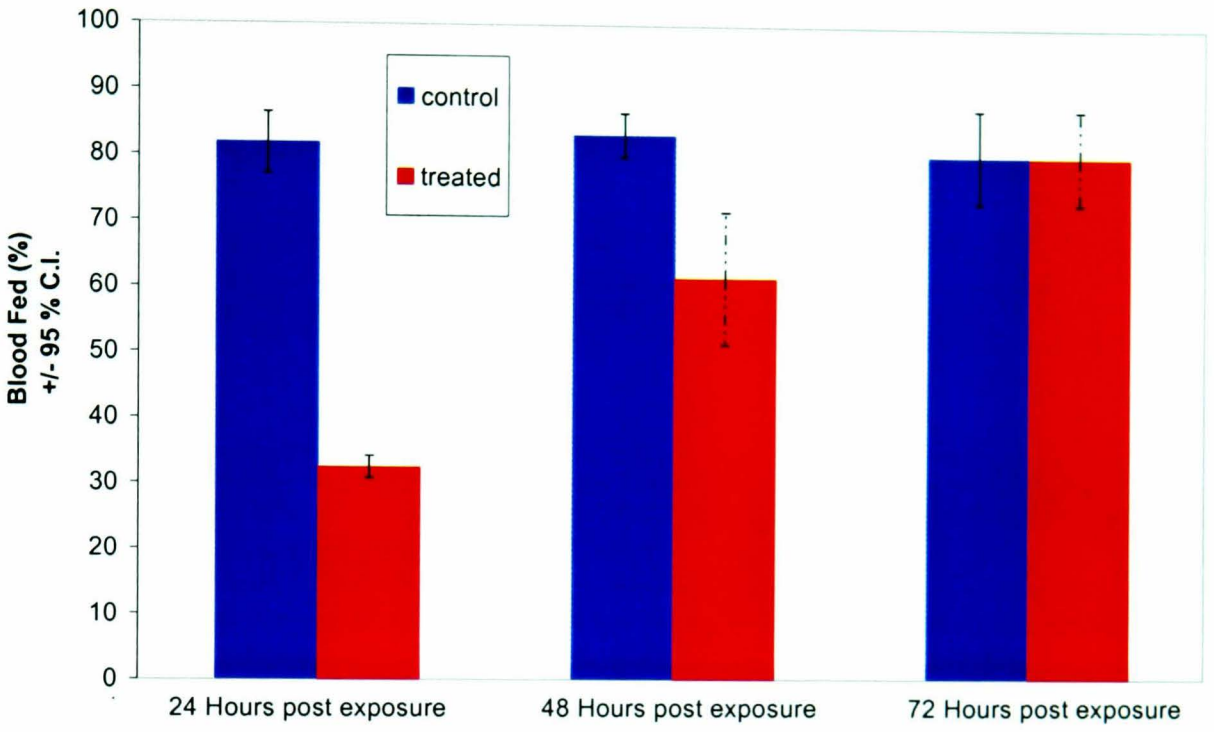
6.3.3. Analysis

Percentage feeding rates in the control and treatment groups (± 95% confidence limits) are shown in figures 20 and 21. Statistical significance of differences in proportions of females feeding in the two groups were determined using Chi square tests. As there may be differences in baseline nutritional status and hunger between different batches of mosquitoes used, data from replicates cannot validly be pooled. For this reason, Mantel-Haenszel Chi square tests were used, with a separate 2 x 2 table for each replicate, comparing the proportion fed / proportion unfed in the control and treatment groups and amalgamation of the results of each table to give a χ^2_{MH} value with one degree of freedom.

6.4. Results

6.4.1. Host seeking behaviour

Figure 20. Effect of permethrin on host seeking behaviour 1, 2 and 3 days post-exposure.



24 hours - Proportion feeding at 24 hours post exposure ;

χ^2_{MH} = 165.75 (df=1) $p < 0.0001$ indicating a highly significant reduction in feeding among the treated females compared with corresponding controls.

48 hours - Proportion feeding at 48 hours post exposure ;

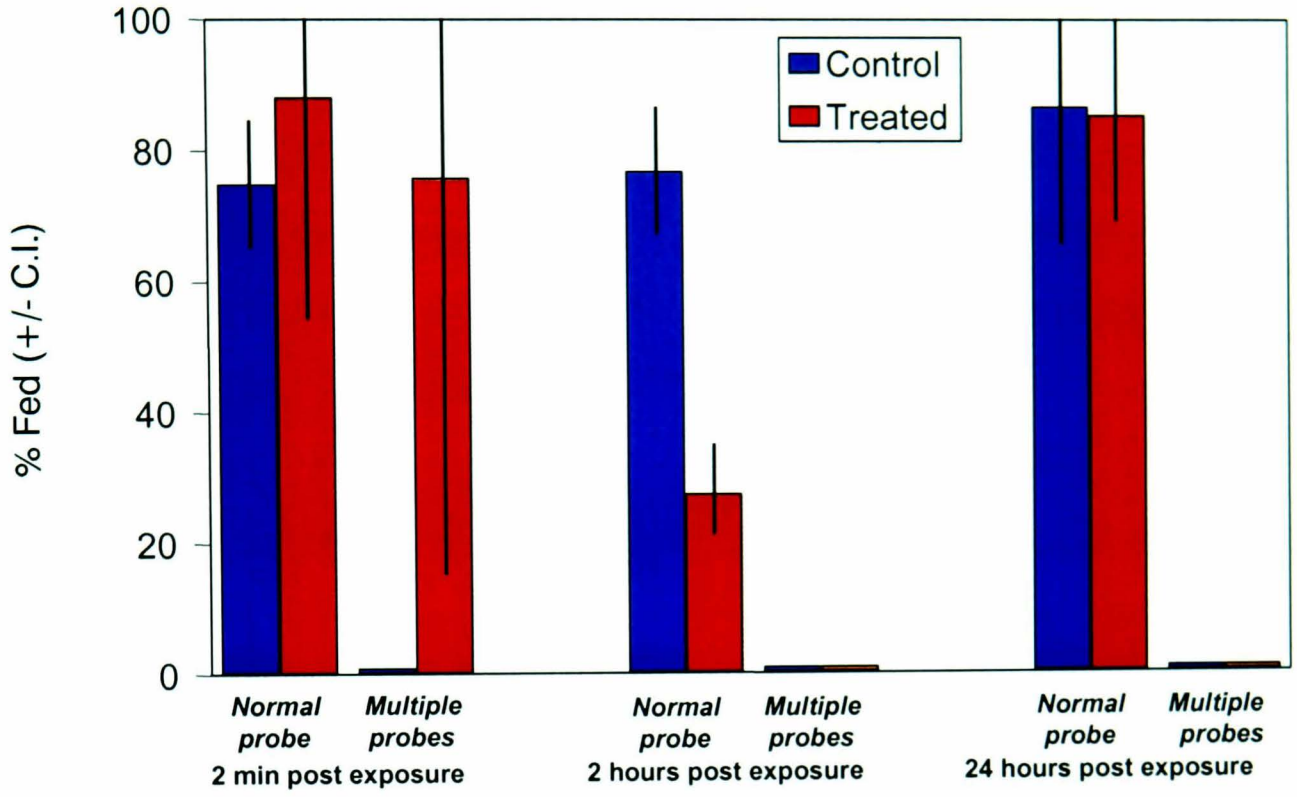
χ^2_{MH} = 27.29 (df=1) $p < 0.0001$ indicating a highly significant decrease in feeding within the treated group compared to the control.

72 hours - Proportion feeding at 72 hours post exposure ;

χ^2_{MH} = 0.03 (df=1) $p = 0.873$ indicating no significant difference in proportion feeding in control or treated groups.

6.4.2. Probe response

Figure 21. Effect of permethrin exposure on probing response.



2 minutes post-exposure - Proportion probing / feeding

χ^2_{MH} = 0.12 (df=1) p = 0.733

indicating no significant difference in probing response following exposure to permethrin.

2 hours post exposure - Proportion probing / feeding

χ^2_{MH} = 20.86 (df=1) $p < 0.0001$

indicating a highly significant reduction in probing even after 2 hours post exposure.

24 hours post exposure - Proportion probing / feeding

χ^2_{MH} = 0.00 (df=1) p = 0.959

not significant, indicating that effects on probing response are transient.

6.5. Discussion

6.5.1. Host seeking

A transient effect of host-seeking and subsequent feeding is observed in figure 20. The most marked reduction was at 24 hours, with only 32.5 % ± 1.7 % of exposed females feeding, compared to 81.8 % ± 4.7 % in the controls. By 48 hours post-exposure, the percentage of treated females feeding had risen to 61.5 % ± 10.1 % with the controls still significantly higher at 83.2 % ± 3.4 %. However, by 72 hours there was no difference in proportion feeding in either group at 80.2 % ± 7.2 %.

The observed reduction in the percentage of females attracted to, and feeding upon, a human host following sublethal permethrin exposure would be beneficial in field situations. It suggests that when individual mosquitoes survive contact with an impregnated bed net, for example, they would then be unlikely to be diverted directly onto an unprotected alternative host, thus causing the bed net to give added protection to the population as a whole, rather than solely the individual under that particular net. Effects of this kind have been detected in experimental hut trials in Tanzania by Lines *et al.* (1987). The fact that the host-seeking behaviour is seen to be only temporarily affected is interesting, with a high level of suppression shortly following contact, and a gradual return to normal over a period of 2-3 days. This period, however short, would expose the females to the additional chance of mortality in the field during which time they would be less likely to seek a host and consequently acquire or pass on an infection. These results may explain the observations of Foil *et al.* (1991) working on marked tabanid flies feeding on bullocks sprayed with a pyrethroid. This group reported a significant delay in those flies having fed and survived contact with treated hosts when subsequently recaptured in baited canopy traps. As yet the host seeking behaviour is not fully understood in mosquitoes, but it appears to be a combination of many responses to a variety of stimuli controlling host recognition, attraction and the final 'homing in' and landing. One possible mode of action

of this phenomenon could be the well documented effects of the pyrethroids on the motor neurons, which would be initially repetitively firing, probably followed by blocking. Without the ability of coordinated flight towards a host, any seeking behaviour would be severely limited by such neuro-physiological disruption.

6.5.2. Probing response

The behaviour which is initiated once a host has been reached by a female is the insertion of the proboscis, known as probing. By removing the need for individuals to seek a host by placing them directly upon one, it was possible to observe the probing response in isolation and deduce that permethrin contact was having an effect upon it. In the initial period immediately following exposure, the probing response was found to be heightened in such a way as to induce prolonged periods of rapid and repetitive insertion and removal of the proboscis, which in some cases never led to a full feed. Although there was no significant difference in the overall proportion feeding in the control and treated groups, 74.9 % ± 9.5 % and 88.2 % ± 35.1 % respectively, it was found that the majority, 75.9 % ± 66.1 %, of the treated females had fed following an abnormally high number of probing attempts. There were no cases of multiple probing in the controls. This could be due to the hyper-excitation of the sensory nervous system which is a characteristic of early pyrethroid poisoning, resulting in the abnormal perception of feeding initiation stimuli. Again, as in the case with host-seeking, the effects were apparently transient. By 2 hours post exposure there were no cases of multiple probing in treated females, but there was now a pronounced reduction in the number of exposed females feeding, 27.6 % ± 4.8 %, compared to 76.9 % ± 7.6 % in controls. At 24 hours post-exposure, effects on proportion feeding and the occurrence of multiple probing was no longer evident, with blood taken by 86.7 % ± 11.2 % of controls and 85.4 % ± 8.7 % of treated females. How this abnormal repetitive probing soon after exposure may affect parasite transmission is questionable. On the one hand, an increase in the number of times a female probes could

be expected to result in an increase in the number of sporozoites being injected due to more saliva being introduced and by prolonging the duration of a feeding attempt. It has been demonstrated by Kelly & Edman (1992), for example, that multiple transmission of *Plasmodium gallinaceum* by *Ae. aegypti* to several hosts can occur by induced serial probing. In these laboratory experiments, infected mosquitoes were allowed to probe on chicks for 10 seconds then held for 1 minute before exposure to a second and third bird. Although numbers of infective hosts were low there were several occasions when infection was passed to a second bird and a few infections occurred even in the third chick probed. There has similarly been one recorded case of malaria transmission to 2 individuals by a single mosquito under field conditions, albeit in a slightly artificial situation. In this instance, researchers were sitting opposite each other collecting *An. flauviatilis* in a typical man landing catch experiment when one particular mosquito observed to bite both people before being caught. Kulkarni & Panda (1984) report that the mosquito was subsequently found to be sporozoite positive and 12 days later both collectors came down with falciparum malaria having been blood-smear parasite free prior to that point. Rossignol *et al.* (1984) even suggest that sporozoites induce lesions in specific parts of the salivary glands of infected mosquitoes in order to prolong the duration of intradermal probing, thus increasing chances of successful transmission. On the other hand, an opposing view would be that repeated probing would serve only to rapidly deplete the readily available supply of sporozoites within the salivary glands. Detailed electron microscopy studies of salivary glands suggest passage of sporozoites from much of the tissue to the central ducts is severely restricted (Wright, 1969, Sterling *et al.*, 1973). Such physical factors could explain the results of Rosenberg *et al.* (1990) which indicate most *An. stephensi* eject *P. falciparum* sporozoites only at the beginning of salivation. The authors suggest that this is due to the fact that only those parasites within the common or secondary salivary ducts are available for uptake. This view would also account for Vandenberg's (1977) estimation that only 1 % of the viable *P. berghei* sporozoites within the salivary glands of

An. stephensi were injected during a feed on rodent hosts. Such a phenomenon would go some way to explaining the apparent discrepancy between calculated rates, calculated from the number of sporozoite positive mosquitoes, and the often lower incidence rates in humans (Molineaux & Gramiccia, 1988). If it is the case that increased probing does not necessarily increase innoculum size, there would be little if any detrimental effect on transmission dynamics as a result of this abnormal probe phenomenon following contact with pyrethroids. With time this excitation disappears, and is replaced by a suppression of the probing response as recorded two hours post contact (Figure 21). The reduced behavioural activity during this period is most probably due to the blocking of nerve impulses within the peripheral nervous system, which is often accompanied by this type of decline in general activity. The suppression is found to be temporary, as by 24 hours the treated mosquitoes are once again initiating the feeding response normally. When considered in combination with the reduced host seeking behaviour described above, it leads to the conclusion that sublethal exposure to permethrin has an important, if temporary, effect on the subsequent feeding of mosquitoes. This in turn may support the usefulness of impregnated bed nets as a community protective measure, even if not all the human population can be given such nets.

CHAPTER 7 Effects on Vector / Parasite interactions

7.1. Introduction

7.1.1. External factors affecting infection rates

A range of unexpected factors have been reported to be associated with transmission dynamics of insect vector-borne diseases. Devaney & Lewis (1993) report that a strain of *Aedes aegypti* susceptible to *Brugia pahangi* became refractory at temperatures of 37°C and above. Mosquito size affects the amount of blood taken during a meal (Ichimori, 1989b), although work by the same author (Ichimori 1989a) reported no effect on *Plasmodium yoelii nigeriensis* oocyst numbers in *Anopheles stephensi* females of different sizes. However, Grimstad & Haramis (1984) found that smaller mosquitoes transmitted La Crosse virus at a much higher rate than larger individuals, presumably as they were weaker and less able to resist infection. By trapping, measuring and dissecting field caught *Anopheles gambiae* in Tanzania, Lyimo & Koella (1991) found *Plasmodium falciparum* sporozoite infections were most prevalent in mosquitoes of intermediate size. Such a phenomenon is most readily explained by a combination of higher survival by larger, uninfected individuals, and a low survival rate of females with high oocyst numbers. Vaughan *et al.* (1994a) working with cultured parasites of *P. falciparum* in laboratory colonies of *An. gambiae* found that if females had taken a previous blood meal prior to an infective feed then fewer oocysts developed, compared to previously unfed individuals. This was attributed to a faster onset of digestion in the former, which resulted in a lower conversion of ookinetes to oocysts. A number of insect pathogens or commensal organisms have been shown to modify infection rates, the common microsporidian parasite *Nosema algerae* being perhaps the most widely reported (Fox & Weiser, 1956; Vavra & Undeen, 1970; Hulls, 1971; Ward & Savage, 1972) In each case *Nosema* infections resulted in a reduction in normal parasite transmission. Similar reductions in malaria infection rates have been reported in mosquitoes infected with *Chlorella* (Chunina

et al., 1991) or *Plistophora* (Garnham, 1956). In one interesting account, Bird (1972), detected Cytoplasmic Polyhedrosis virus multiplying within developing oocysts of *Plasmodium yoelii* resulting in malformation of sporozoites. In the sandfly vector *Phlebotomus papatasi,* Schlein & Jacobson (1995) found that the diverse source of meals taken by these insects could influence development of the human parasite *Leishmania major.* In areas with large numbers of chicken and turkey rearing sheds there were much lower infection rates than in the surrounding areas, a fact put down both to the direct mortality that this type of blood causes on the parasite as demonstrated *in vitro,* and to the long term reduction in infective potential of avian fed flies. This work in the field corroborated their findings in the laboratory (Schlein & Jacobson, 1994) which found that haemoglobin inhibited production of chitin, and thus development, of *L. major in vitro.* As well as host derived inhibitory factors, the same authors found that some plant sugar feeding under field conditions resulted in plant derived antimicrobial agents in the sandfly crop which were lethal to parasites (Schlein & Jacobson, 1995).

7.1.2. Effects of insecticides on infection rates

In recent years there have been reports that contact with insecticides at doses not high enough to kill vectors may have some effect on the parasites they carry. Carle *et al.* (1986) first described an apparent reduction in the infectivity of the vector, *An. stephensi,* with rodent malaria, *P. yoelii yoelii.* This group concluded that sublethal exposure to the pyrethroid deltamethrin resulted in fewer females acquiring an infection and a reduced development of oocysts and sporozoites. The treatment dose caused around 50 % mortality, and the relatively small number of individuals used in the experiments along with marked variation amongst replicates made the conclusions not entirely convincing, however, it did serve to raise an interesting line of further investigation. The authors go on to suggest the effects on infection were caused by direct toxicity of the compound to the parasite. Whilst testing a range of biologically active substances on the development of

P. gallinaceum in *Ae. aegypti*, Chunina *et al.* (1991) reported that the pyrethroid, cypermethrin, at doses which led to 40 % larval mortality resulted in significant reduction in the sporozoite index of surviving females. Despite these positive findings, work by Prasittisuk & Curtis (1982), found no effect at all of DDT, malathion or dimilin on the susceptibility of several strains of *An. gambiae* or *An. stephensi* to *P. yoelii nigeriensis*, which suggests it may be a feature of pyrethroids only.

Apart from direct toxicity effects on parasites within the vector, it would seem possible that insecticides could modify the often complex interactions which take place between the two organisms. Work on the vector of *Trypanosoma cruzi*, *Rhodnius prolixus*, carried out by Rembold and Garcia and reported by Beard (1989), demonstrates this phenomenon caused by low doses of the insecticide azadirachtin derived from the neem tree. This substance does not cause direct mortality on the parasite in culture but, if added to an infected feed, it prevents the multiplication of the parasite within the gut and thus stops the transmission of the organism to a future host. Early work by Garcia *et al.* (1984) investigated the molecular biology of the interaction between *T. cruzi* and *R. prolixus*. Having identified a number of biochemical markers, in a range of parasite clones, they suggest that the insect gut environment was involved in selecting specific strains of the parasite which multiply and mature successfully. A few years later Garcia (1988) reported that azadirachtin interfered in some way in this host / parasite interaction to block development and infection. The fact that azadirachtin did not inhibit growth of the parasite in culture, and the fact that transmission blocking could be partly reversed by ecdysone, led the group to suggest an indirect action on gut biochemistry, although no candidate mechanisms were offered at that time. Garcia & Azambuja (1991) later went further in their investigations, dismissing the possibility of parasite damage by gut proteases, and finding no alteration in sensitive gut physiological markers which may account for the unfavourable habitat theory. Although there is still little clear evidence pointing to a

specific mode of action, it seems likely that transmission is blocked due to changes in the host neuroendocrine system.

7.1.3. Aims of this chapter

Having reviewed the factors which can influence development of parasites within their specific vector it appears that sublethal exposure to pyrethroids may have an effect on infection of mosquitoes. This study used rodent malaria, *P. yoelii nigeriensis* in a susceptible strain of *An. stephensi,* as a model to determine whether contact with pyrethroids can influence oocyst development in the vector. Experimental methods used included exposure before and after infective feed, and a more realistic method of taking an infective feed through a treated net. The aim was to compare oocyst rates in treated and control groups. Outcomes are discussed in relation to possible influence on malaria vector control programmes using these compounds.

7.2. Materials

7.2.1. Mosquitoes

BEECH	*Anopheles stephensi*
DUB/APR	*Anopheles stephensi*

7.2.2. Parasites

N67	*Plasmodium yoelii nigeriensis* A rodent malarial parasite described by Killick-Kendrick (1973). Held in liquid N_2 at LSHTM

7.2.3. Insecticides

Permethrin	0.25 % on paper
Permethrin	250 mg/m² on polyester netting
Deltamethrin	250 µg/m² on polyester netting
Lambda-cyhalothrin	250 µg/m² on polyester netting

7.2.4 Chemicals

Mercurochrome Product No. M7011. Supplied by Sigma Ltd, UK

7.3. Methods

To determine how sublethal doses of various pyrethroids affected the ability of females to acquire an infection of rodent malaria, treated and control groups were given infective feeds combined with exposure in one of several different ways. In an attempt to relate these results to human disease transmission, a number of experiments were subsequently carried out using a strain of human malaria (see chapter 8)

7.3.1. Rodent infective feeds

Mouse erythrocytes parasitised with *Plasmodium yoelii nigeriensis* (strain N67) were routinely held under cryopreservation at LSHTM. To prevent loss of infectivity during continual *in vitro* culture, as described by Wery (1968), they are cyclically transmitted through mice and mosquito hosts before returning to liquid nitrogen. Parasitised blood was taken from nitrogen storage and held at 37°C for 10 minutes in a waterbath. PBS containing 15 % glucose was added to the blood which was inoculated intraperitoneally (i.p.) at a rate of 0.2ml / mouse. From day 3 onwards tail snips were taken from these mice until thin smears, methanol fixed and Giemsa stained, showed a parasitaemia of 15 - 20 %. At this point blood from these donor mice was collected by cardiac puncture using a heparinised syringe and inoculated i.p. into a second group of mice at a rate of approximately 10^6 parasitised red blood cells / mouse. These mice were again tail snipped to record level of infection from day 3 onwards until a parasitaemia of 15 - 20 % was reached. Hypnorm was used following manufacturer's instructions to anaesthetise the parasitised mice which were then used in subsequent infective mosquito feeding experiments.

7.3.2. Determination of infection / oocyst rate

A standard midgut dissection technique utilising mercurochrome stain, as originally recommended by Eyles (1950), was used. After an infective feed all females were held at 24°C ± 2°C and 75-80 % humidity for 10 days, and individuals were then anaesthetised with chloroform. Midguts were removed with the aid of a dissecting microscope at x4 using spade-end mounted needles. A drop of 2 % mercurochrome was placed on a clean glass slide, the female was laid on its side and a small nick made with a needle between the 6th & 7th segment of the abdomen. While holding the thorax with one needle, the second needle was placed near the end of the abdomen and, with a steady movement, drawn away. In this way the midgut is pulled clear from the integument and can be isolated and then covered gently with a cover slip. Oocysts take up the orange stain and can be clearly seen and counted using a microscope at x40 or x100. Numbers of oocysts < 50 were counted individually, whilst heavier infections were categorised into the following groups : 50-100, 100-200, 200+. Plates 1 and 2 show typical examples of high and low oocyst burdens in mosquito midgut preparations.

7.3.3. Infection prior to sublethal contact with permethrin

Females of the BEECH strain of *An. stephensi* were offered an infective feed when 4 days old and those which had engorged were divided into two random groups and then exposed to either permethrin or an oil control in WHO bioassay tubes. After 1 hour it was noticed that in these blood fed insects the usual partial mortality observed in unfed mosquitoes following treatment with the sublethal permethrin dose was greatly reduced. However, no increase in exposure time or dose was used. Batches of between 20 and 30 females were used in each of 5 replicates of both groups.

7.3.4. Exposure to permethrin prior to infection

Batches of randomly selected four day old BEECH females underwent treatment with permethrin or oil control and were then allowed to recover for 24 hours before being offered an infective feed. Any individual not having taken a full feed was discarded. Oocyst dissection occurred at day ten as detailed above. Five replicates of 20 to 30 females in both treated and control groups were used.

7.3.5. Infective feed through an impregnated net

Females of the permethrin resistant DUB/APR strain were used in these experiments as this was the only strain capable of completing and surviving a full feed through the impregnated netting. Three-day-old, mated, females were allowed to feed on an infective host mouse covered by either control or pyrethroid impregnated polyester netting at the predetermined concentration. This experiment simulates the field situation with mosquitoes feeding on a person resting against a treated bednet. Only females completing a full feed to repletion were retained for the experiments, with unfed or part fed individuals being discarded to overcome possible effects of blood meal size on infection rates. In each experiment batches of between 20 and 30 females were used in each of 5 replicates of both groups.

7.3.6 Statistical analysis

As each replicate of the infection experiments occurred on different days there is a likelihood that the gametocyte levels in the host rodents will be different in each case. As this is an important factor in subsequent mosquito infection rates it is necessary to use a method of analysis which will take this inter-replicate confounding variable into account. For this reason, a Mantel-Haenszel Chi Square test was chosen as a stratified analysis as it compares each replicate separately and then pools their contribution to a Chi Square value (Kirkwood, 1988). In each case the infection rates from each replicate are also presented

in summary tables shown as number infected / total number (e.g. 10 / 20 = 50 % infection). χ^2_{MH} value and the corresponding Relative Risk (RR) parameter, ± 95 % confidence limits, were computed using the STATCALC facility in EPI INFO 6.0.

Mean and 95 % confidence intervals were calculated using arc sine transformed percentage infection rate data from each of the replicates.

Plate 1. **Mosquito midgut with heavy oocyst burden (200 +)**

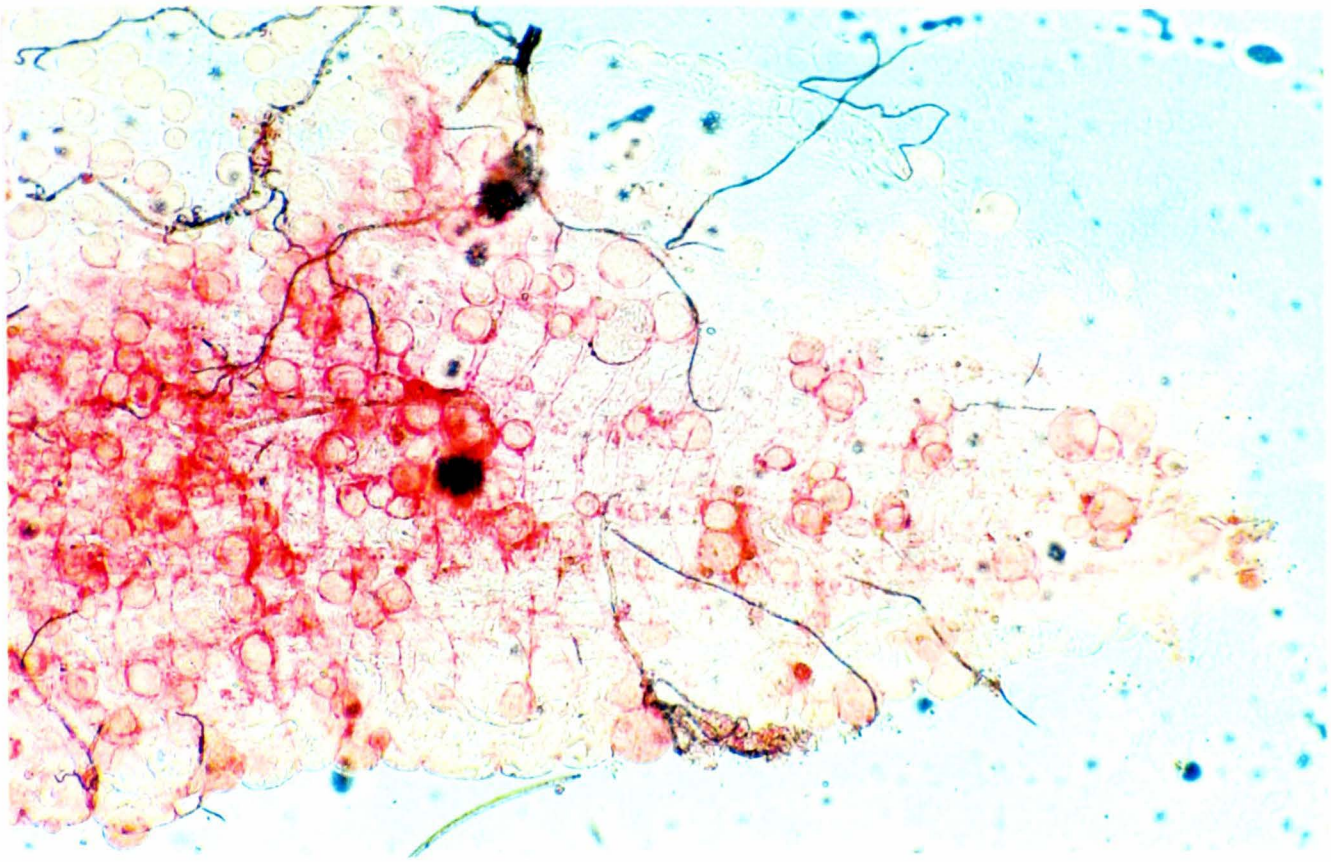
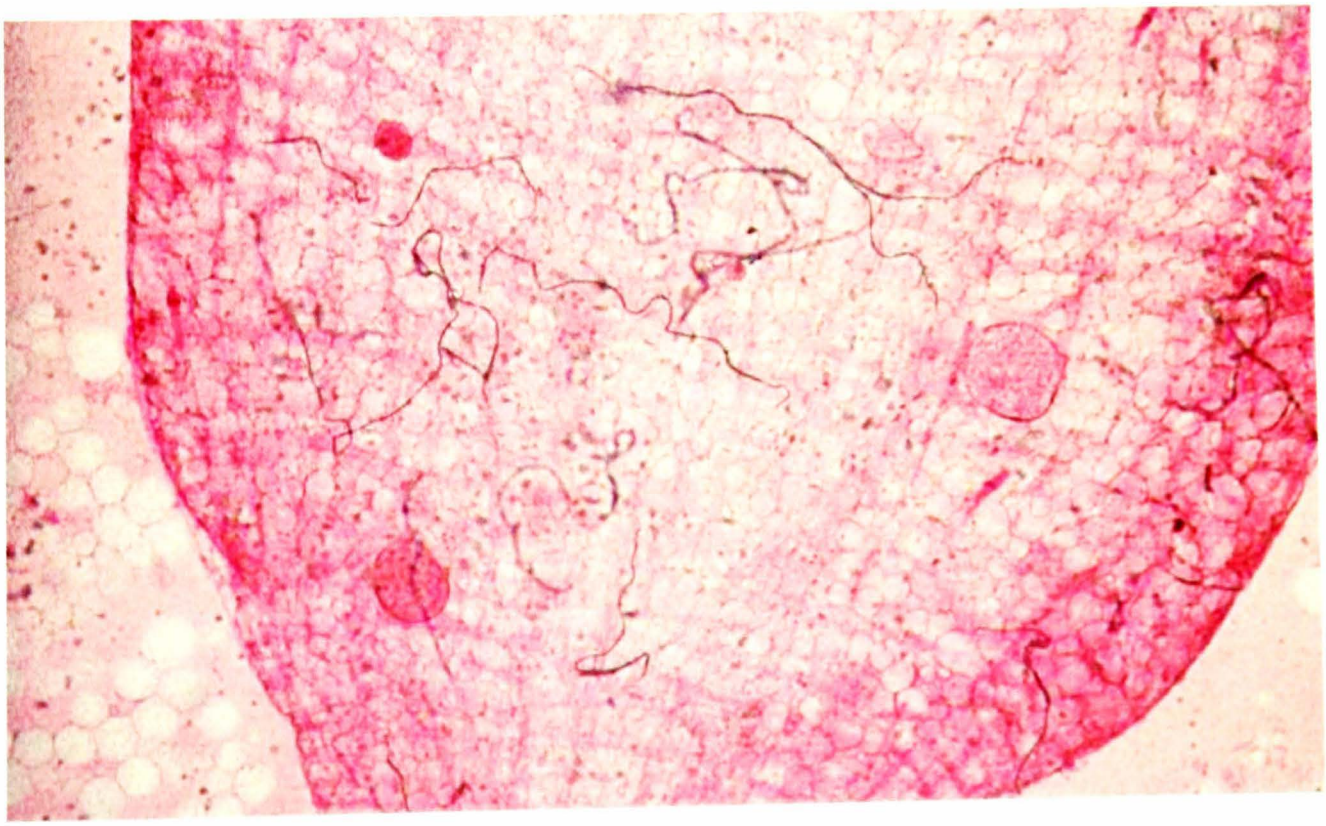


Plate 2. **Midgut with a low oocyst burden (< 10)**



7.4. Results

7.4.1 Infection prior to sublethal contact with permethrin

Levels of infection (% ± 95 % confidence intervals) observed in control and treatment groups are presented in figure 22. Mantel-Haenszel Chi square stratified analysis of 5 tables showed a significant reduction in the treatment group :

Table 8. Infection prior to sublethal contact with permethrin
Number of females infected / total.

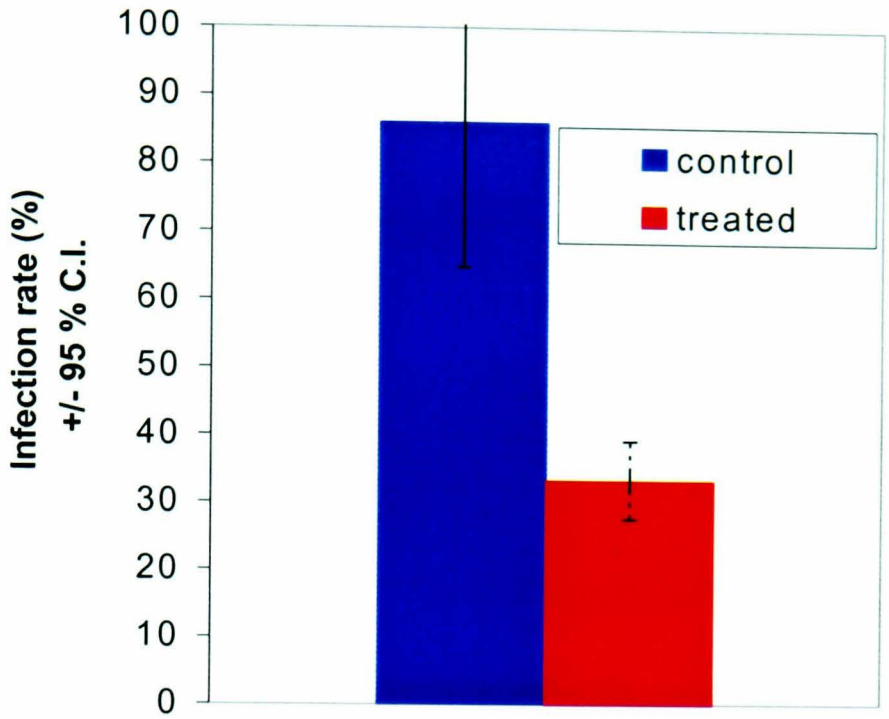
Replicate	Control	Treated
1	11 / 16	8 / 28
2	15 / 16	8 / 24
3	19 / 21	9 / 25
4	9 / 10	5 / 14
5	14 / 17	7 / 21

$\chi^2_{MH} = 47.10$ (df=1) $p < 0.0001$ indicating a highly significant reduction in infection prevalence in treated females compared to the controls.

RR = 0.39 (0.30 – 0.52)

Infection prevalence in controls was 86.1 % ± 21.4 % compared to 33.4 % ± 5.8 % in permethrin exposed individuals.

Figure 22. **Effect of permethrin on *P. yoelii* oocyst infection levels in *Anopheles stephensi* with exposure occurring shortly after infective feed.**



7.4.2. Exposure to permethrin prior to infection

Percentage infection rates for the treatment and control groups are shown in figure 23. This histogram demonstrates not only that significantly fewer individuals develop an infection, but that the oocyst burden is also higher in midguts of unexposed females. Mantel-Haenszel Chi square stratified analysis of 5 tables showed a significant reduction in the treatment groups as follows ;

a) Overall infection levels :

Table 9. **Exposure to permethrin prior to infection - Overall number of females infected / total.**

Replicate	Control	Treated
1	13 / 15	3 / 5
2	15 / 16	21 / 29
3	18 / 20	14 / 21
4	14 / 15	13 / 20
5	19 / 21	20 / 27

χ^2_{MH} = 11.9 (df=1) p = 0.0006 indicating a significant reduction in infection prevalence in treated females compared to the controls. RR = 0.76 (0.65 – 0.88).

Infection prevalence in controls was 90.8 % ± 2.9 % compared to 67.6 % ± 5.7 % in permethrin exposed individuals.

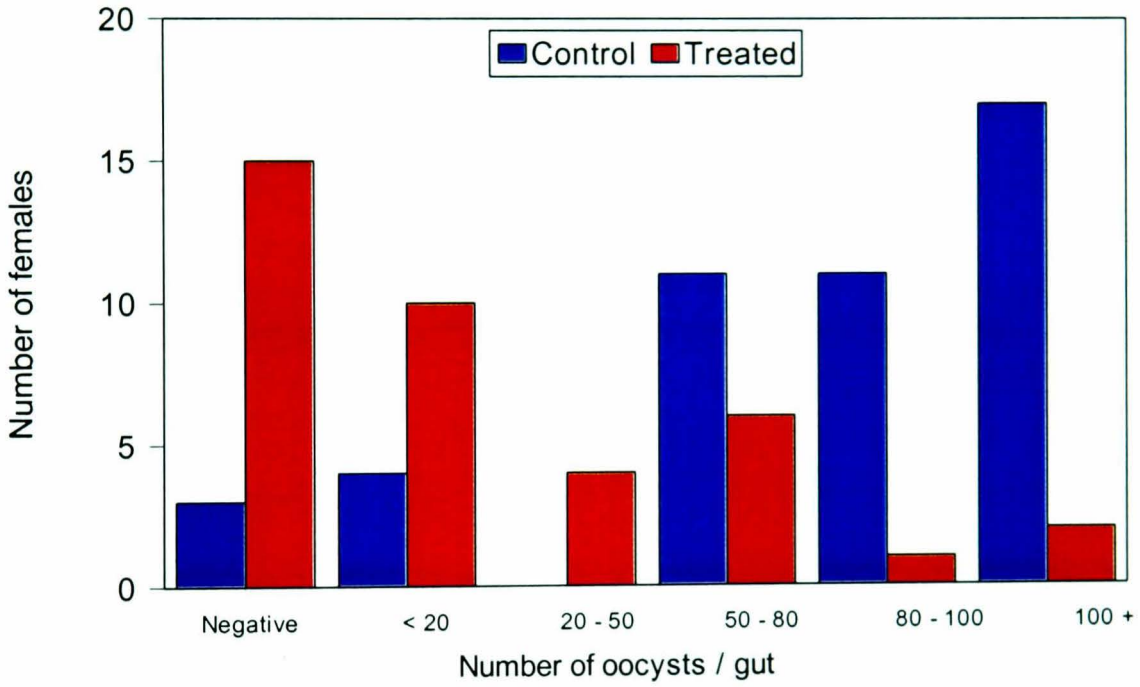
b) Greater than 20 oocysts/ gut :

Table 10. **Exposure to permethrin prior to infection - Number of females infected with greater than 20 oocysts / gut.**

Replicate	Control	Treated
1	13 / 15	1 / 5
2	15 / 16	8 / 29
3	18 / 20	5 / 21
4	14 / 15	8 / 20
5	18 / 21	13 / 27

χ^2_{MH} = 50.5 (df=1) $p < 0.0001$ indicating a highly significant reduction in infection prevalence in treated females compared to the controls. RR = 0.40 (0.31 – 0.53). Prevalence of 20 or more oocysts in controls was 89.8 % ± 3.7 % compared to 34.8 % ± 14.9 % in permethrin exposed individuals.

Figure 23. **Effect of permethrin on *P. yoelii* oocyst infection levels in *Anopheles stephensi* with exposure occurring shortly before infective feed.**



7.4.3. Infective feed through a permethrin -impregnated net

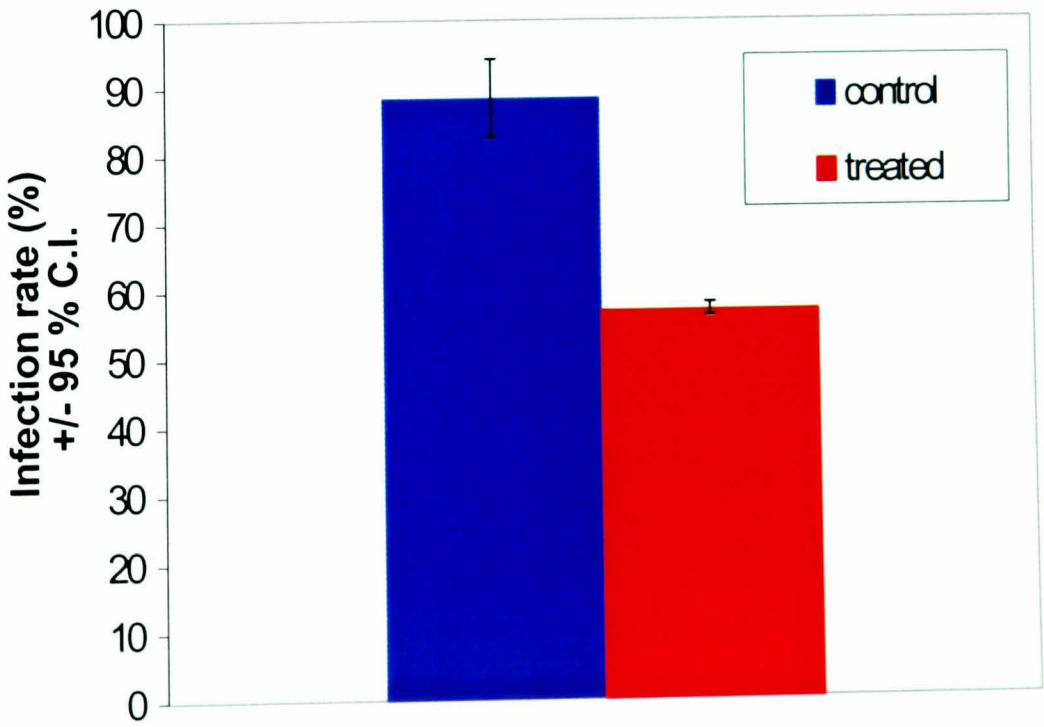
Infection rates in treatment and control groups are presented in figure 24. Mantel-Haenszel Chi square stratified analysis of 5 tables showed a significant reduction in the treatment group :

Table 11. **Infective feed through a permethrin-impregnated net - Number of females infected / total.**

Replicate	Control	Treated
1	17 / 20	10 / 18
2	23 / 25	12 / 20
3	20 / 21	11 / 18
4	19 / 23	10 / 19
5	17 / 20	10 / 18

χ^2_{MH} = 23.0 (df=1) $p < 0.0001$ indicating a significant reduction in infection prevalence in treated females compared to the controls. RR = 0.65 (0.54 – 0.78). Infection prevalence in controls was 88.4 % ± 5.8 % compared to 57.0 % ± 0.9 % in permethrin exposed individuals.

Figure 24. **Effect of permethrin on *P. yoelii* oocyst infection levels when exposure occurs whilst taking a feed on an infective host through impregnated netting.**



7.4.4. Infective feed through a deltamethrin impregnated net

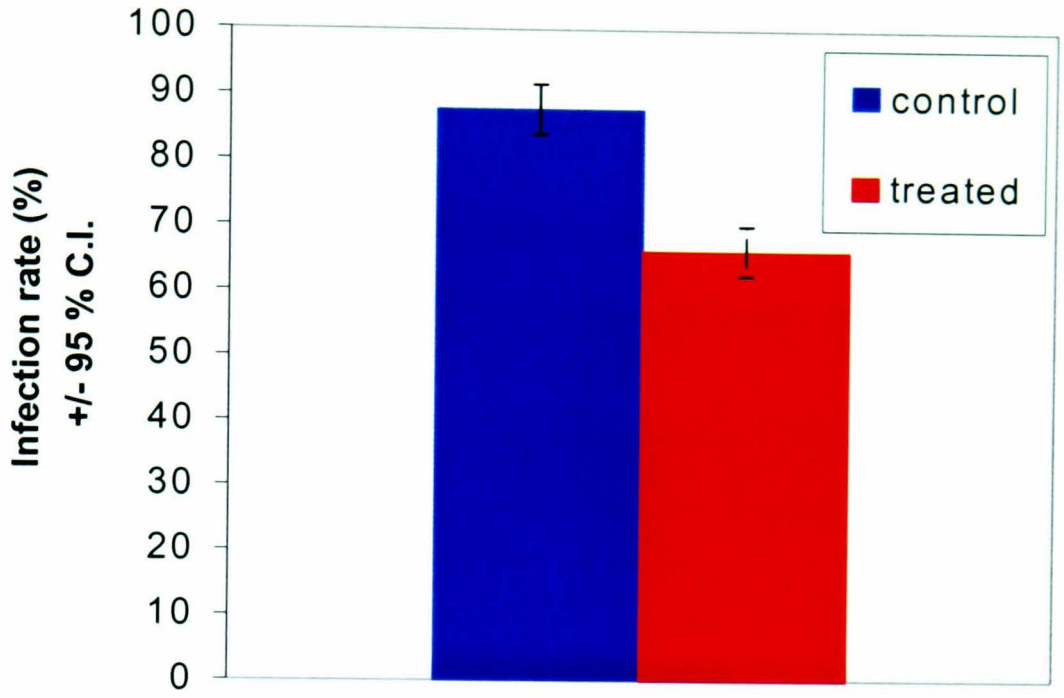
Overall infection rates in treatment and control groups are presented in figure 25. Mantel-Haenszel Chi square stratified analysis of 5 tables showed a significant reduction in the treatment group :

Table 12. Infective feed through a deltamethrin-impregnated net - Number of females infected / total.

Replicate	Control	Treated
1	23 / 25	12 / 20
2	18 / 20	12 / 20
3	16 / 20	13 / 20
4	18 / 20	14 / 20
5	17 / 20	15 / 20

$\chi^2_{MH} = 12.1$ (df=1) $p = 0.0005$ indicating a significant reduction in infection prevalence in treated females compared to the controls. RR = 0.75 (0.64 – 0.88). Infection prevalence in controls was 87.7 % ± 3.9 % compared to 66.2 % ± 3.8 % in deltamethrin exposed individuals.

Figure 25. **Effect of deltamethrin on *P. yoelii* oocyst infection levels when exposure occurs whilst taking a feed on an infective host through impregnated netting.**



7.4.5. Infective feed through a lambda-cyhalothrin impregnated net

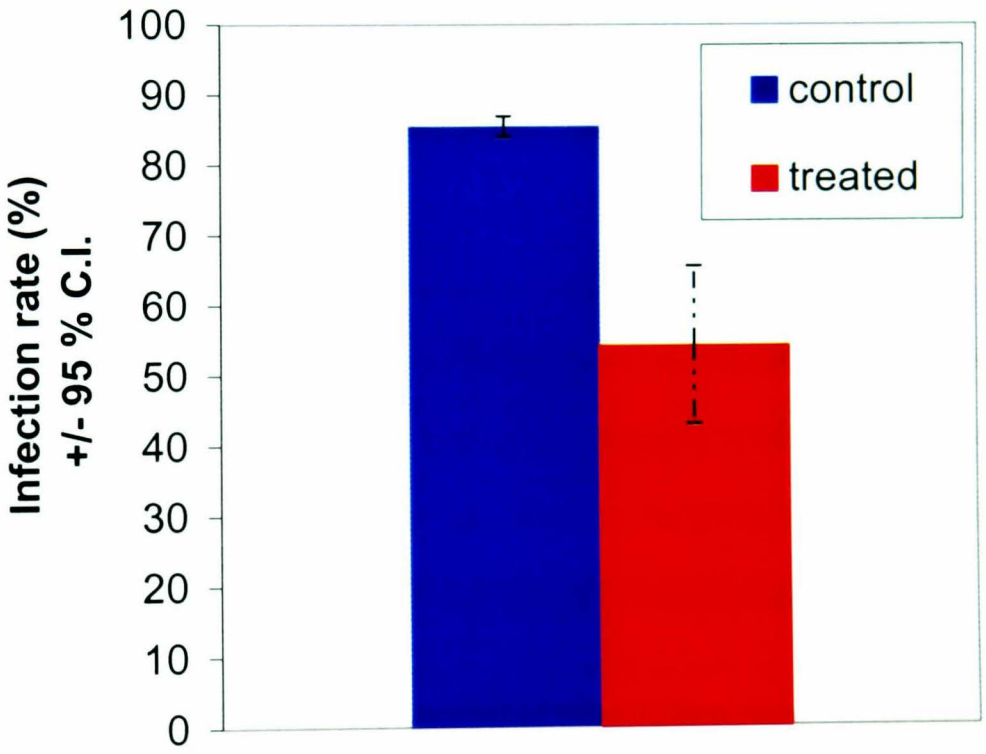
Infection rates in treatment and control groups are presented in figure 26. Mantel-Haenszel Chi square stratified analysis of 5 tables once again showed a significant reduction in the treatment group :

Table 13. **Infective feed through a lambda-cyhalothrin-impregnated net - Number of females infected / total.**

Replicate	Control	Treated
1	17 / 20	10 / 20
2	18 / 22	9 / 17
3	18 / 20	6 / 16
4	17 / 20	12 / 20
5	17 / 20	14 / 20

χ^2_{MH} = 20.4 (df=1) $p < 0.0001$ indicating a significant reduction in infection prevalence in treated females compared to the controls. RR = 0.64 (0.52 – 0.78). Infection prevalence in controls was 85.4 % ± 1.5 % compared to 54.2 % ± 11.3 % in lambda-cyhalthrin exposed individuals.

Figure 26. **Effect of lambda-cyhalothrin on *P. yoelii* oocyst infection levels when exposure occurs whilst taking a feed on an infective host through impregnated netting.**



7.5 Discussion

Findings presented in figures 22 – 26 give a clear demonstration that sublethal contact with a range of synthetic pyrethroids results in significant inhibition of parasite development within mosquitoes. Moreover, the actual timing of the contact with the chemical appears

not to be limited to a very short period at the time of gametocyte uptake; definite effects were recorded following exposure before, during and after the infective feeding process. Similar levels of reduction were seen with each of the three compounds tested on netting, with 57 % becoming infected after contact with permethrin (controls 88%), 66 % infected with deltamethrin (controls 87%) and 54 % with lambda-cyhalothrin (controls 85 %). These results are very encouraging in the context of using pyrethroid insecticides in malaria vector control programmes. One could speculate that, if similar inhibition of human *Plasmodium* species within their natural mosquito vectors could be demonstrated, this phenomenon may play an important role in control of disease transmission without necessarily affecting easily visible vector control parameters such as man biting rates and mosquito density. If this is true, field studies which use such entomological factors in isolation as a measure of success of interventions, would need to include additional techniques, such as oocyst or sporozoite rates such as those used by Magesa *et al.* (1991), Curtis *et al.* (1998b), and Maxwell *et al.* (1999).

The results in figure 22 showing a significant inhibition of oocyst development in females having had a sublethal exposure shortly following an infective feed, are the most pronounced. This is likely to be due to the fact that parasites in the fresh blood meal, just initiating their sporogonic development, will be subjected to the maximal effects of the pyrethroid exposure. It is therefore not unexpected that the next series of experiments, where insecticide exposure occurs prior to an infective feed shows a slightly more limited effect. In this case, the reduction in the overall number of mosquitoes becoming infected, whilst still significant between the treated and control groups, is less than that in the earlier group. However, it is interesting to note in figure 23, that there is a very obvious reduction in the number of treated insects with higher oocyst loads. This demonstrates that the permethrin is still exerting a regulatory influence over the development of the parasite even if infection does ultimately still occur in the midgut. Even this more modest

effect of limiting the burden of oocysts may prove to have an effect in transmission of disease. Although there are a number of theories as to the factors which determine the length of prepatent period, duration and severity of malaria attacks, and indeed the development of immunity in nature, there are many who believe this may be due to sporozoite load or quality injected during a bite (Boyd, 1939; McGregor, 1964; Vanderberg, 1977; Ungureanu *et al.*, 1977; Rosenberg *et al.*, 1990; Greenwood *et al.*, 1991; Beier, 1993; Pumpuni *et al.*, 1997). If such fundamental factors are indeed attributable to the sporozoite innoculum size, then any factor which reduces the number of oocysts would presumably be important. Sinden (1987) suggested that each mature oocyst may release up to 10,000 sporozoites into the haemocoel to migrate to the salivary glands, and single oocyst dissections of field caught *An. dirus* from Thailand by Rosenberg & Rungsiwongse (1991), found a mean of 3,688 sporozoites of *P. vivax* or 3,385 of *P. falciparum*. With such high numbers resulting from each individual oocyst, any reduction in their numbers developing in the gut could make a considerable difference to sporozoite loads in the mosquito. In addition, there is a general consensus amongst groups working in the field that there are relatively low sporozoite numbers in the majority of naturally infected vector species (McGregor, 1964; Pringle, 1966; Beier *et al.*, 1991; Kabiru *et al.*, 1997). A positive correlation has been reported between oocyst number and gland infection (Pringle, 1966; Rosenberg *et al.*, 1990), which suggests that the effect of pyrethroids to reduce the number of oocysts per gut may prove beneficial in epidemiological terms, even if the overall prevalence of infected mosquitoes is not significantly decreased.

The results presented in figures 24, 25 and 26 of reduced parasite development in females which had taken an infective feed through an impregnated net are particularly pertinent in the context of current malaria control strategies. Most authoritative works agree that the most promising method of sustainable malaria control in the tropics is

through the implementation of an impregnated bed net programme (Curtis *et al.*, 1990; Rozendaal, 1989 & 1997; Lengeler, 1998). Pyrethroid impregnated bed nets have been proven to have marked effect on natural vector populations (Magesa *et al.*, 1991; Jawara *et al.*, 1998; Maxwell *et al.*, 1999), to significantly reduce mortality and morbidity (D'Alessandro *et al.*, 1995 & Nevill *et al.*, 1996) and are cost effective and sustainable within the community (Lengeler *et al.*, 1996; Aikins *et al.*, 1998).

One of the biggest potential problems, which may lessen the efficacy of such nets, is likely to be the development of pyrethroid resistance in the vector population. Resistance to pyrethroids has already become a very serious problem in control of many important agricultural pests (McCaffery, 1998), where these compounds are used heavily. There are a growing number of reports of pyrethroid resistance in vector mosquitoes from around the world (Elissa *et al.*, 1993; Vulule *et al.*, 1994; Darriet *et al.*, 1997). As yet the situation in malaria vectors is less severe than that seen in many agricultural pests, probably due to the highly efficient and fast kill of heterozygotes by pyrethroids on nets (Hodjati & Curtis, 1997), and the fact that their public health use, particularly on bed nets, promotes contact only with mated females seeking a blood meal, placing no selection pressure on the males, and thus diluting any resistance genes in the population as a whole (Curtis *et al.*, 1993). Despite this, concern quite rightly remains, and various methods are being investigated to delay the development of resistance and to seek alternative candidates for use on bed nets (Curtis *et al.*, 1998a). One of the earliest reports in anophelines was of low levels of pyrethroid resistance in wild *An. stephensi* from Dubai, which was subsequently increased to much higher levels through prolonged selection pressure in the laboratory (Ladonni, 1988). More worrying were reports by Vulule *et al.*, (1994), which suggested that the use of permethrin-impregnated bed nets in Kenya were responsible for reduced susceptibility of *An. gambiae* in bioassays. However, when the same group conducted similar studies in the same area a couple of years later, they found no further

increase in resistance (Vulule *et al.* 1996). In this case, one could argue that this resistance had not been caused by the use of impregnated bed nets, as these had continued to be used in the intervening years and so would have continued selection pressure leading to increasing levels of resistance. The earlier results may have been due to variation in bioassay procedure, as Hodjati & Curtis (1996) have demonstrated an age dependant effect of pyrethroid tolerance. The most encouraging signs come from studies in parts of China where impregnated bed nets have been in use for many years, yet the vector population of *An. sinensis* has shown no increase in KT_{50} or LT_{50} (Kang *et al.*, 1995). In Côte d'Ivoire, however, several authors have demonstrated *An. gambiae* have developed resistance to pyrethroids (Elissa *et al.*, 1993; Darriet *et al.*, 1997), which Martinez-Torres *et al.* (1998) have identified as being facilitated by a knockdown resistance (*kdr*) altered target site (Na^+ channel) mechanism. Unlike DDT, whose environmental use has been heavily restricted in recent decades, pyrethroids are used widely on crops, and it is considered likely that such cases of resistance in mosquitoes have arisen as a result of agricultural use and not impregnated bed nets (Curtis *et al.*, 1998). Whilst there continues to be such large populations of pyrethroid susceptible vector populations, and low numbers of heterozygotes in endemic areas, it is highly unlikely that mosquitoes will be able to feed on a host through an adequately impregnated net and survive (Hossain & Curtis, 1989). However, when resistance develops to a more significant degree in the future, the results of the present study would be expected to make most impact. If it were proven that parasite development was inhibited in mosquitoes having fed on an infected person sleeping under an impregnated net, then the sublethal pyrethroid exposure during the act of feeding may act to block further transmission. In this way, impregnated nets may still have an important role to play in malaria control even if their current efficacy at providing personal protection is lessened by resistance.

CHAPTER 8 Effects on mosquito infectivity of *Plasmodium falciparum*

8.1. Introduction

Having established that sublethal contact of mosquitoes with a range of pyrethroids, either before, during or following an infective feed, can reduce infectivity using a rodent malaria model (chapter 7), it is important to determine whether this is the case for parasites of humans. Although rodent models are ideal for conducting fundamental research into mammalian host / parasite relationships and basic biological processes, there is clearly disparity between the two systems. The previous chapter, along with work of numerous other researchers, has shown particularly large numbers of oocysts developing within the midgut of mosquitoes fed on rodent gametocytaemic blood (Vaughan *et al.*, 1994; Ichimori, 1988; Kurihara & Kikuchi, 1991). Unlike the 100's – 1,000's of oocysts reported in such cases, the number of *P. falciparum* oocysts developing, both in laboratory experiments (Ponnudurai *et al.*, 1982; Ranford-Cartwright *et al.*, 1991) and in the field (Pringle, 1966; Billingsley *et al.*, 1994; Babiker *et al.*, 1994) are constantly at least an order of magnitude smaller, averaging around 5 oocysts per gut (Lyimo & Koella, 1992). Whether this large discrepancy is due to a greatly reduced intrinsic infectivity or quantity of gametocytes imbibed by the insect, or in higher losses during development in the insect vector is unclear, but it does mean that results obtained through rodent models cannot be considered indicative of effects within the human malaria parasites. Despite this problem, one might be encouraged by the fact that vector oocyst numbers are so low in the majority of natural human malaria infections that there is the potential for any similar reduction to have considerable impact. During early work in East Africa, Pringle (1966) dissected large numbers of wild caught *An. gambiae* and *An. funestus*, and found sporozoite rates of 3.2 - 3.6 % and 0.9 – 1.4 %, and oocyst rates of 4.2 – 7.2 % and 2.3 – 3.3 %, respectively. Most significantly, he found that the majority of all infected mosquitoes of both species

had only a single oocyst, and virtually all had less than 5. In addition, it was found that the average number of sporozoites were only 2,000 – 4,000, which led Pringle to conclude "...it would also appear that infections of this order are chiefly derived from the maturation of a single oocyst or, at most, two oocysts". Similar conclusions were drawn from work in West Africa by McGregor (1964), who again found natural sporozoite loads were relatively small. This author then goes on to point out that this is of relatively little importance to the parasite itself, as each individual sporozoite injected results in but a single schizont, but each of these in turn can liberate some 30,000 merozoites ! With such low oocyst numbers found in natural vector populations it is encouraging to believe that even minor factors which impede development of the parasite could have a profound effect on ultimate vectorial capacity. Both of these groups working in the field in Africa studied mosquito sample sizes far in excess of the minimum acceptable as derived by the mathematical models of Billingsley *et al.* (1994), who investigated the relationship between prevalence and intensity of *P. falciparum* in natural mosquito populations. A comprehensive review of *Plasmodium* development in the vector is provided by Beier (1998), which not only covers the developmental processes which occur, but also presents findings from many field studies investigating oocyst burden and prevalence in relation to malaria transmission.

8.1.1. Aims of this chapter

Having established previously in the chapter 7 that sublethal exposure of the vector to pyrethroids can reduce both individual infection rates and oocyst burden, this study aims to expand the investigation to determine whether similar effects are observed with human malaria. To achieve this, the rodent model used earlier was replaced with cultured human malaria parasites, *P. falciparum*. The aim was to make comparisons between oocyst infection rates in groups of females sublethally exposed to permethrin or a control. Findings are discussed in relation to potential effects on disease transmission and vector control activities.

8.2. Materials

8.2.1. Mosquitoes

DUB/APR *Anopheles stephensi*

8.2.2. Parasites

3D7 *Plasmodium falciparum* A clone of strain NF54 isolated in The Netherlands by limiting dilution

8.2.3. Insecticides

Permethrin 0.25 % on paper

Permethrin 250mg/m² on polyester netting

8.2.4. Chemicals

PABA Para-aminobenzoic acid (4-aminobenzoic acid) 99% Free acid. Product No. A9878. Supplied by Sigma Ltd, UK

RPMI Standard human blood cell culture medium. Product No. R6504. Supplied by Sigma Ltd, UK

Hypoxanthine Product No. H9636. Supplied by Sigma Ltd, UK

8.3. Methods

8.3.1. *Plasmodium falciparum in vitro* culture

Plasmodium falciparum (strain 3D7) was held in routine culture in the Institute of Cell, Animal and Population Biology at the University of Edinburgh using the technique described by Ifediba & Vanderberg (1981). Cultures were initiated at 0.2 % parasitaemia and 12 % haematocrit in RPMI-1640, 15 % human serum and 50$\mu g/ml^{-1}$ hypoxanthine. 20 ml cultures in fresh human erythrocytes were held at 37°C in an atmosphere of 3% O_2,

2% CO_2 and 95 % N_2. Medium was changed daily until day 12 - 14 when these mature cultures were used to re-seed new gametocyte cultures.

8.3.2. Membrane feeding

Cultures of *Plasmodium falciparum* 3D7 parasites containing mature gametocytes were centrifuged. The resultant pellets were resuspended in 3 volumes of uninfected human red blood cells with sufficient human serum to give a 50% haematocrit. This suspension was introduced into a glass membrane feeder as described by Rutledge *et al.* (1964), which was then lowered onto the top of a netting covered cardboard cup which held the mosquitoes. Approximately 25 three day old mated females were in each pot and had been starved for the preceding 24 hours. Following the infective feed all mosquitoes were held under secure conditions at 26°C and 70 - 80% relative humidity. A food source on cotton wool pads was provided daily consisting of 5% glucose plus 0.05% PABA solution, as recommended by Vanderberg & Gwadz (1980).

8.3.3. Infective feed through an impregnated net

Groups of approximately 30 hungry 3 day old females of the pyrethroid resistant DUB/APR strain were allowed to take an infective membrane feed until repletion through either a piece of permethrin impregnated netting or a control net. All mosquitoes were then held under secure insectary conditions for between 10 & 11 days prior to dissection and oocyst counts.

8.3.4. Infection prior to sublethal dose

Two random groups of hungry 3 day old DUB/APR females were presented with an infective membrane feed of the cultured *P. falciparum*. One of these groups was immediately given a 30 minute exposure to 0.25 % permethrin papers in WHO test kits.

whilst the other received an equivalent period on a silicone oil control paper. All mosquitoes were held under secure insectary conditions for 10 - 11 days prior to dissection.

8.3.5. Infection rate and oocyst numbers

On day 10 or 11 post infective feed the mosquitoes were anaethetized with chloroform, then placed singly in watch glasses containing saline to ensure they would not escape, before they were removed from the secure insectary. Each female was then dissected as described in chapter 7 although the much lower oocyst rates meant that actual numbers were recorded in each case, unlike in the case of rodent infections which could only be allocated into abundance ranges.

8.4. Results

8.4.1 Effects on infective feed through permethrin impregnated netting

In this series of experiments there was an unexpectedly high mortality (75 – 80 %) in the treatment group even though a highly permethrin resistant strain of mosquito was used. An additional problem encountered was very low feeding rates (average 20 %) due to a combination of increased probing activity and a lower avidity of mosquitoes for feeding on a membrane rather than a live host. All these factors resulted in fewer than 20 individuals completing an infective feed surviving to day 10 for dissection in a total of 6 replicates, too few for a meaningful comparison to be made. For this reason it was decided to switch methods to one where permethrin exposure was in WHO tubes following an infected feed.

8.4.2. Effects of permethrin exposure after infective feed

The results of the 5 replicates are presented as histograms in figure 27. Although the control infection rates varied to some degree in each replicate the corresponding treatment infection rates were consistently lower in each instance. The variation in control infection levels can be explained by the fact that each replicate was conducted several weeks apart

and a fresh gametocyte culture was used in each case. As infectivity is influenced by many factors, particularly the parasite culture used, a number of confounding variables are introduced which make direct comparison between replicates problematical. For this reason the results of each replicate were tested for significance using the Mantel-Haenszel Chi Square test, a stratified analysis which takes any confounding variables between replicates into account (Kirkwood, 1988), as described earlier in chapter 7.

Table 14. ***P. falciparum*** **infection prior to sublethal contact with permethrin - Number of females infected / total.**

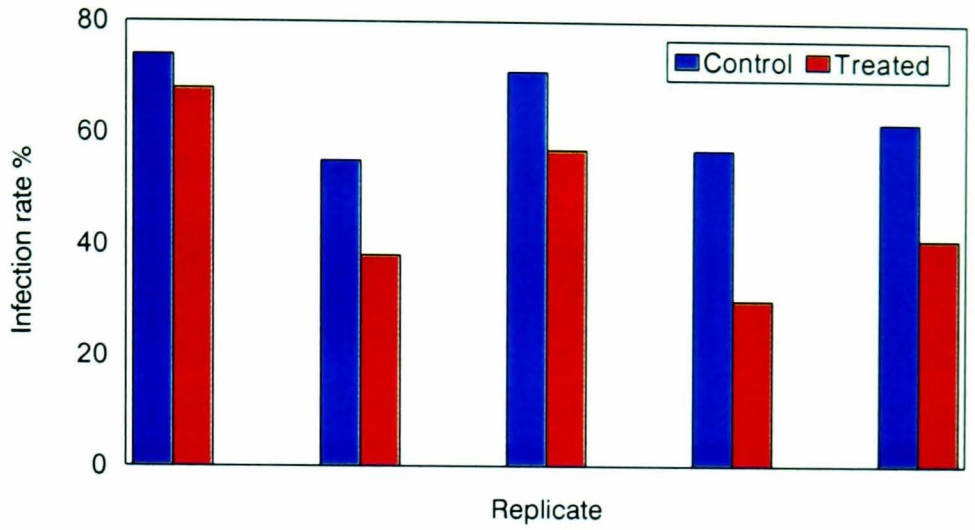
Replicate	Control	Treated
1	12 / 17	13 / 23
2	12 / 22	13 / 34
3	20 / 27	28 / 41
4	12 / 21	10 / 33
5	26 / 42	16 / 39

$$\chi^2_{MH} = 7.81 \ (df=1) \qquad p = 0.005$$

$$RR = 0.74 \ (0.60 - 0.90)$$

This indicates that sublethal contact with permethrin after the infective feed resulted in a significant reduction in development of *P. falciparum* in *An. stephensi*. Mean infection prevalence rate in treated individuals was 46.4 % ± 18.9 % compared to 64.0 % ± 6.0 % in controls.

Figure 27. **Effect of permethrin on *Plasmodiom falciparum* oocyst infection levels in *Anopheles stpehensi* where exposure was shortly after the infective feed. Infection rates from individual replicates.**



8.5. Discussion

The early experiments (8.4.1), which compared females which had taken an infective feed through impregnated netting, did not yield sufficient data to compare infection rates between treatment and controls. Although reduced infection rates had earlier (chapter 7) been demonstrated with permethrin used in this way it must be remembered that this was using live rodents infected with malaria which result in much higher numbers feeding. In addition, increased probing caused by the insecticide, plus a common reduction in feeding avidity under *in vitro* membrane conditions, may well have resulted in prolonged exposure which bought about the increased mortality observed. In the experiments described in chapter 7 using *P. yoelii,* mortality was lower, the overall mosquito infection rates were very high and oocyst loads per individual were much larger. In this study using *P. falciparum,* the low feeding rates combined with the high mortality has resulted in it being impossible to identify any reduction which may have been bought about by the treatment in this way.

It was for this reason that a further series of experiments were conducted, this time using WHO permethrin exposure papers. In this way it was hoped that the contact with the insecticide could be increased by prolonging the exposure time, and thus heighten any effect on infection to a point that is detectable using *P. falciparum*. Using this method, there was a significant ($p = 0.005$) reduction in the development of the parasite within the vector from 64 % ± 6.0 % in controls to 46.4 % ± 18.9 % following exposure. The histogram in figure 27 shows that in each replicate there were fewer infected mosquitoes in the group which had received a sublethal dose of permethrin compared to the controls. This is a very encouraging result, with potential impact on the use of pyrethroid insecticides in the field for vector control programmes, as the number of infected mosquitoes in a vector population is a key factor in determining transmission dynamics (Service, 1993). The use of pyrethroid insecticides in the field of malaria control has been increasing for many years, whether for residual house spraying (Rishikesh *et al.*, 1978), impregnated bed nets (WHO, 1989; Rozendaal, 1989; Curtis *et al.* 1990; Lengeler *et al.*, 1996) or other forms of personal protection such as soaps and repellents (Yap, 1986; Curtis *et al.*, 1987; Lindsay and Janneh, 1989; Lindsay *et al.*, 1998), treated clothing (Schreck *et al.*, 1978), tent treatment in refugee camps (Hewitt *et al.*, 1995) or room fumigants such as mosquito coils (Hudson & Esozed, 1971). Based on our findings here it seems likely that sublethal contact with these substances may have an influence, not only on physiology of the insect, but on its ability to develop and transmit the disease itself. There are several field studies which report a reduction in the number of *P. falciparum* sporozoite positive individuals in vector populations following introduction of pyrethroid impregnated bed nets (Magesa *et al.* 1991; Curtis *et al.* 1998b; Maxwell *et al.* 1999). Given the results shown in the present study, it is possible that some of this reduction in sporozoite index could be the result of inhibition of parasite development in females sublethally exposed to the pyrethroids being used.

CHAPTER 9 Relationship between infection and treatment timing

9.1. Introduction

9.1.1. Identification of developmental stages affected by permethrin

Having demonstrated an effect of sublethal doses of pyrethroids on malarial infections a number of studies were considered in an attempt to elucidate the mechanisms involved. The sequence of events which occurs between the time when a mosquito takes an infective feed, and the subsequent establishment of a discernible infection is fairly well understood. Thus, by varying the time at which insecticidal treatment occurs in relation to the time of the infective feed it may be possible to pin point those processes within this host / parasite interaction which are affected by the insecticide. Such experiments may show whether a particular stage of the parasite life cycle is susceptible to direct toxic effects of the insecticide. Alternatively, disruption in any of the phases of the host / parasite interaction which are highly sensitive to microenvironmental abnormalities may be identified, such as exflagellation, ookinete development / migration or oocyst establishment. Changes in the normal mosquito gut environment at such times may be the key to this phenomenon. Thus, identifying the periods at which infection can be disrupted may identify which are the most likely targets. In the case of inhibition of *Trypanosoma cruzi* development within its vector, *Rhodnius prolixus*, by azadirachtin (Garcia, 1988) it was found that a single treatment resulted in the failure of that individual insect to acquire subsequent infections throughout the rest of its life (Garcia *et al.*, 1991). If the action of permethrin on malarial infections in mosquitoes is similar, this could be demonstrated by allowing subsequent infective feeds at intervals after an initial sublethal exposure.

9.1.2. Important stages during establishment of an infection

There are a number of potential targets during the early development of the malarial parasite once it has entered the mosquito host. The first two days see several substantial

changes occurring within the parasite itself, as well as a number of host mediated factors, all of which may influence the success of any subsequent infection. Within the first few minutes of an infective meal being imbibed, gametocytes undergo maturation into micro- or macro-gametes and exflagellation begins (Bruce-Chwatt, 1980). Once formed, the zygote begins to elongate and the motile ookinetes form rapidly. Vaughan *et al.* (1994) reported the largest numbers appearing at around 8 hours in *An. stephensi*. The same authors have shown that, in many anopheline species by 12-24 hours post-feeding, the majority of ookinetes have traversed the forming peritrophic membrane and begun entering the gut wall. Huber *et al.* (1991) reported that the ookinete invasion of the midgut epithelial cells begins at 24 hours but that the major invasion takes place around 30 hours. From this point on, the developing oocysts grow on the outer surface of the stomach wall, until mature sporozoites have developed within them and are finally liberated when the oocyst bursts. The exact mechanisms and regulatory features of many of the developmental changes outlined above will be investigated in greater detail in subsequent chapters. It is clear, however, that, with so many transformations taking place, there is wide scope for suppression of one or more processes by permethrin, which may explain this compound's inhibition of normal infection rates.

9.1.3. Aims of this chapter

Earlier chapters have shown that sublethal exposure of vectors to pyrethroids can reduce subsequent malaria development in that individual. This study aimed to begin the process of elucidating how such a phenomenon occurs. When one considers that there are a multitude of early developmental processes during the first few hours and days which may influence the establishment of the parasite within the vector, it is clearly important from the outset to narrow the possibilities down as much as possible. The following work was designed to fulfil this aim by conducting a series of time-course experiments, where

exposure occurred at pre-set intervals after the infective feed. The intention was to focus in on the most important stages where the insecticide is exerting its action.

9.2. Materials

9.2.1. Mosquitoes

BEECH *Anopheles stephensi*

DUB/APR *Anopheles stephensi*

9.2.2. Parasites

N67 *Plasmodium yoelii nigeriensis*

9.2.3. Insecticides

Permethrin 0.25 % on paper

Permethrin 250 mg/m² on polyester netting

9.3. Methods

9.3.1. Double feeds through treated / untreated netting

DUB/APR females were offered an infective feed through a net impregnated with a pyrethroid or without insecticide. Half of each group, taken in batches of 25 at random, were left for 10 days prior to dissection to determine infection levels. The remaining females, in batches of 25, were then held for 5 days under standard conditions and then offered a second infective feed, but on this occasion both groups fed through an untreated control net. These females where then held for an incubation period of 10 days prior to dissection.

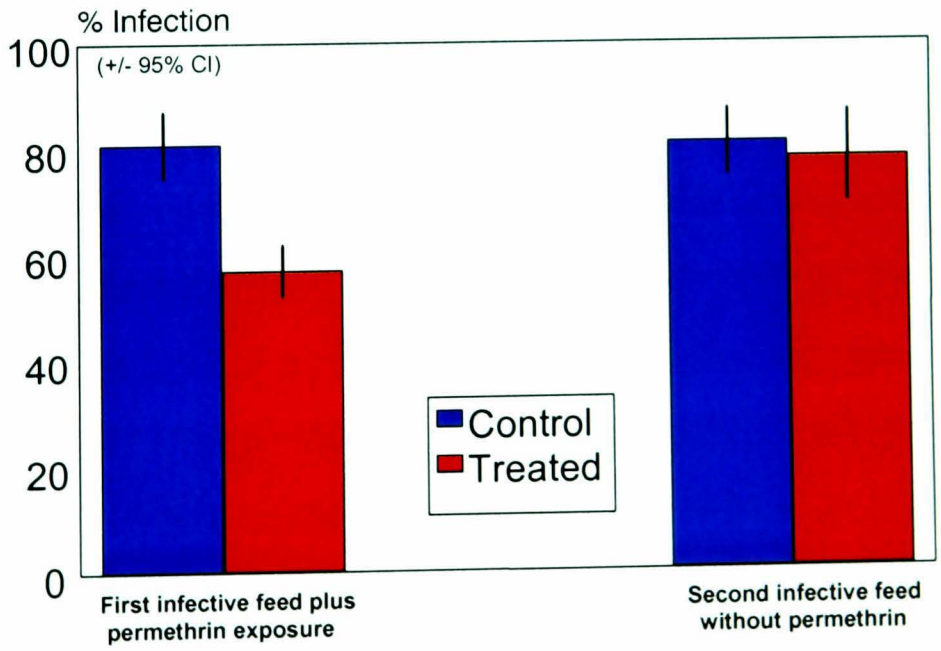
9.3.2. Delayed insecticidal treatment

Batches of 50 females of the BEECH strain were allowed to feed until repletion on an infective mouse host and then held for a predetermined period under standard conditions. After this period half of each group were exposed to a sublethal dose of permethrin in WHO testing tubes whilst the rest were exposed to an oil control. All sets of females were held for a total of 10 days after the infective feed under standard incubation conditions prior to dissection and counting of oocyst numbers. The periods between infective feed and permethrin or control treatments were as follows: 1, 6, 12, 18, 24, 48 and 72 hours.

9.4. Results

9.4.1. Effects of permethrin on subsequent infections

Figure 28. **Effect of sublethal doses of permethrin on *Plasmodium* infectivity in later life - Pooled data from 5 replicates**



Mantel-Haenszel Chi square stratified analysis of 5 tables shows a significant reduction in the treatment group when the infective feed took place at the same time as exposure :

Table 15. **Effect of sublethal doses of permethrin on *Plasmodium* infectivity in later life - Data from first infective feed on number of females infected / total.**

Replicate	Control	Treated
1	15 / 19	10 / 18
2	14 / 17	12 / 20
3	16 / 20	11 / 20
4	16 / 20	12 / 20
5	21 / 25	9 / 18

$\chi^2_{MH} = 12.93$ (df=1) $p = 0.0003$

RR = 0.69 (0.56 – 0.85)

This indicates that following a single infective feed through a permethrin-impregnated net there was a statistically significant reduction in the proportion of females becoming infected compared to untreated controls. Mean infection prevalence rate in treated individuals was 57.0 % ± 2.8 % compared to 81.0 % ± 5.3 % in controls. The relative risk of infection derived from the Mantel-Haenszel Chi square test of 0.69 indicates that untreated females are 31 % more likely to be infected than those exposed to permenthin.

However, similar analysis found no difference between groups when a subsequent infective feed was offered without permethrin exposure at that time :

Table 16. **Effect of sublethal doses of permethrin on *Plasmodium* infectivity in later life - Data from second infective feed on number of females infected / total.**

Replicate	Control	Treated
1	15 / 19	14 / 17
2	14 / 17	12 / 15
3	16 / 20	16 / 22
4	16 / 20	12 / 16
5	21 / 25	12 / 15

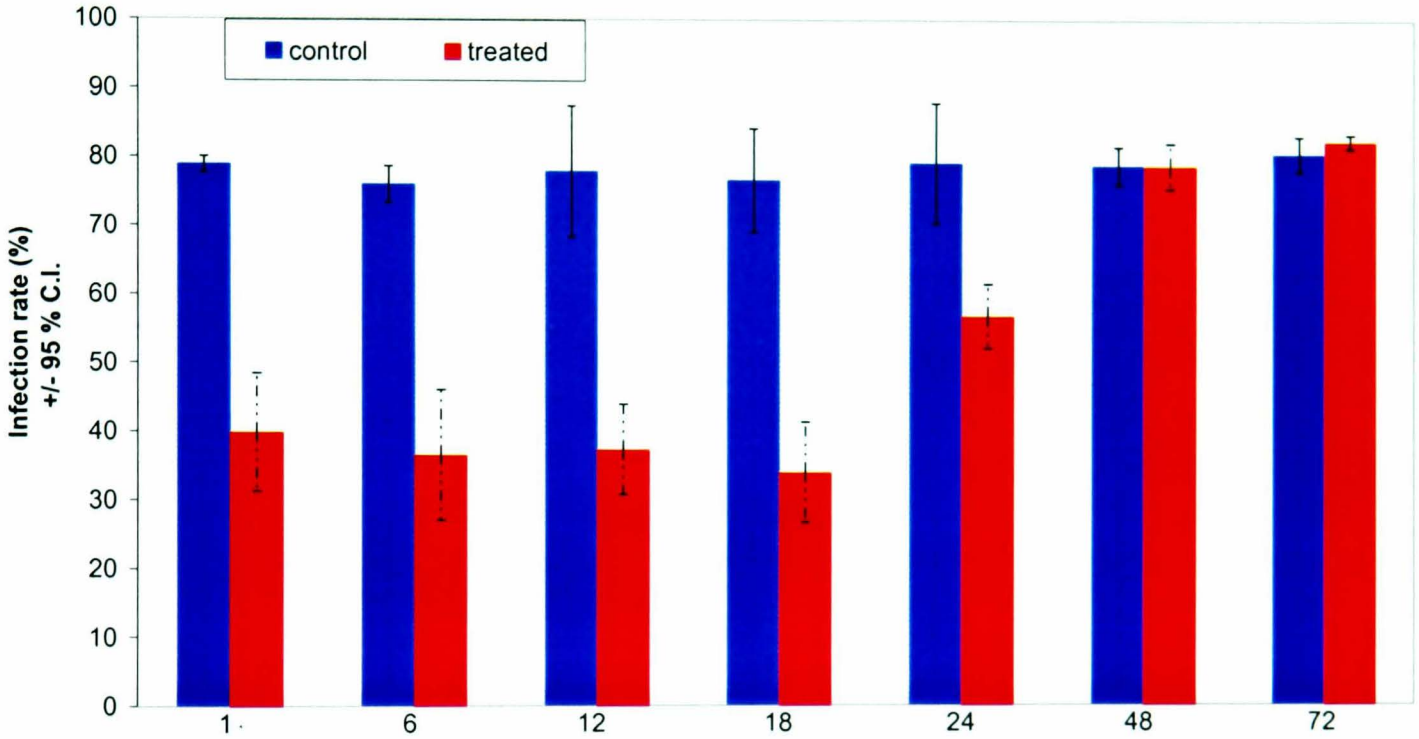
$\chi^2_{MH} = 0.12$ (df=1) $p = 0.727$

RR = 0.96 (0.83 – 1.11)

Indicating that following a second infective feed, this time through a non-impregnated net, there was no significant reduction in the proportion of females becoming infected compared to untreated controls. Mean infection prevalence rate in previously treated individuals was 78.0 % ± 7.4 % compared to 81.0 % ± 5.3 % in controls. Relative risk of infection showed almost no difference between groups (RR = 0.96).

9.4.2. Timing of exposure in relation to effects on treatment

Figure 29. **Influence of timing of permethrin exposure on *Plasmodium yoelii nigeriensis* infection rates in *Anopheles stephensi*.**



Mantel-Haenszel Chi square tests, were used to compare infection rates between control and treatment groups at each time point :

1 hour

Table 17. Timing of exposure in relation to effects on treatment.
Data at 1 hour. Number infected / total

Replicate	Control	Treated
1	15 / 20	7 / 19
2	15 / 20	7 / 16
3	16 / 20	14 / 27
4	17 / 21	6 / 22

$\chi^2_{MH} = 22.3$ (df=1) $p < 0.0001$

RR = 0.51 (0.39 – 0.69)

This indicates that there was a significant reduction in females exposed at 1 hour post-infective feed becoming infected compared to untreated controls. Mean infection prevalence in treated individuals was 39.8 % ± 8.6 % compared to 77.8 % ± 1.2 % in controls. Relative risk of infection in the treatment group was 51 % (RR = 0.51), compared to the untreated controls.

6 hours

Table 18. **Timing of exposure in relation to effects on treatment. Data at 6 hours. Number infected / total**

Replicate	Control	Treated
1	17 / 25	9 / 23
2	14 / 18	8 / 17
3	19 / 24	9 / 23
4	18 / 23	4 / 18

$\chi^2_{MH} = 24.2$ (df=1) $p < 0.0001$

RR = 0.49 (0.36 – 0.66)

This indicates that there is a significant reduction in females exposed at 6 hours post-infective feed becoming infected compared to untreated controls. Mean infection prevalence rate in treated individuals was 36.5 % ± 9.5 % compared to 75.9 % ± 2.7 % in controls. Relative risk of infection in the treatment group was 0.49 compared to the untreated controls.

12 hours

Table 19. **Timing of exposure in relation to effects on treatment. Data at 12 hours. Number infected / total**

Replicate	Control	Treated
1	16 / 25	10 / 22
2	13 / 17	7 / 16
3	17 / 20	7 / 20
4	16 / 19	6 / 23

$\chi^2_{MH} = 23.51$ (df=1) $p < 0.0001$

RR = 0.49 (0.36 – 0.66)

This indicates that there was a significant reduction in females exposed at 12 hours post-infective feed becoming infected compared to untreated controls. Mean infection prevalence rate in treated individuals was 37.3 % ± 6.6 % compared to 77.8 % ± 9.6 % in controls. Relative risk of infection in the treatment group was 0.49 compared to the untreated controls.

18 hours

Table 20. Timing of exposure in relation to effects on treatment. Data at 18 hours. Number infected / total

Replicate	Control	Treated
1	16 / 25	9 / 25
2	15 / 20	6 / 17
3	15 / 18	9 / 21
4	14 / 17	4 / 18

$\chi^2_{MH} = 24.8$ (df=1) $p < 0.0001$

RR = 0.46 (0.33 – 0.64)

This indicates that there was a significant reduction in females exposed at 18 hours post-infective feed becoming infected compared to untreated controls. Mean infection prevalence rate in treated individuals was 34.0 % ± 7.3 % compared to 76.6 % ± 7.6 % in controls. Relative risk of infection in the treatment group was 46 % of the untreated controls.

24 hours

Table 21. **Timing of exposure in relation to effects on treatment. Data at 24 hours. Number infected / total**

Replicate	Control	Treated
1	17 / 25	13 / 21
2	17 / 22	10 / 18
3	20 / 25	12 / 19
4	16 / 18	10 / 22

$\chi^2_{MH} = 7.6$ (df=1) $p = 0.0571$

RR = 0.73 (0.58 – 0.91)

This indicates that there was only a borderline significant difference in the proportion of females becoming infected following exposure at 24 hours post-infective feed. Mean infection prevalence in treated females was 56.8 % ± 4.7 % compared to 79.1 % ± 8.8 % in controls. Relative risk of infection in the treatment group was 72 % (58 % - 91 %) of that of the untreated controls.

48 hours

Table 22. **Timing of exposure in relation to effects on treatment. Data at 48 hours. Number infected / total**

Replicate	Control	Treated
1	18 / 25	13 / 18
2	16 / 20	13 / 17
3	18 / 23	15 / 18
4	21 / 25	15 / 18

$\chi^2_{MH} = 0.00$ (df=1) $p = 0.976$

RR = 1.02 (0.87 – 1.19)

Indicating no significant difference in the proportion of females becoming infected following exposure at 48 hours post-infective feed. Mean infection prevalence in treated individuals was the same as that in controls, 78.7 % ± 3.3 % and 78.7 % ± 2.8 %, respectively.

72 hours

Table 23. Timing of exposure in relation to effects on treatment. Data at 72 hours. Number infected / total

Replicate	Control	Treated
1	20 / 25	12 / 15
2	15 / 20	12 / 15
3	24 / 30	17 / 20
4	19 / 22	22 / 26

$\chi^2_{MH} = 0.02$ (df=1) $p = 0.899$

RR = 1.02 (0.89 – 1.18)

Indicating no significant difference in the proportion of females becoming infected following exposure at 72 hours post-infective feed. Mean infection prevalence rate in treated individuals were very similar to that in the controls, 82.3 % ± 1.0 % and 80.4 % ± 2.6%, respectively.

9.5. Discussion

9.5.1. Effect of permethrin on subsequent infections

The fact that permethrin exposure only inhibits the establishment of an infection initiated within a relatively short time interval after the contact is clearly demonstrated by figures 28 and 29. Even in insects which have shown reduced infection rates following treatment a subsequent infective feed results in normal oocyst development. This indicates that the

mechanism by which inhibition is brought about acts either directly upon the parasite in the gut at the moment of exposure or that an essential host / parasite relationship is affected in a transient manner. This is unlike the mode of parasite inhibition exhibited by azadirachtin in triatomine bugs described by Garcia & Azambuja (1991) and Garcia *et al.* (1991). In this case, a single treatment with the chemical not only stops development of *Trypanosoma cruzi* from that particular blood meal but blocks development of the parasite within the gut throughout the rest of the insect's life. The authors explain this remarkable effect by the chemical's action on disruption of the vector's endocrine system and subsequent changes in the normally favourable gut environment.

9.5.2. Influence of exposure timing on infection

From the results presented above in figure 29, and the Mantel-Haenszel Chi Squared tests, it can be seen that permethrin inhibits infection if the exposure occurs 18 hours or less after an infective feed. There also appeared to be some effect on infection rates when exposure occurs as late as 24 hours post infection (figure 30), but the corresponding M-H Chi Square was of borderline significance ($p = 0.057$). This suggests that once development of the parasite passes beyond a certain point the ability of the compound to influence progression of the infection is limited. This critical time of between 18 and 48 hours post feed corresponds to the migration of mature ookinetes through the epithelial cell wall of the midgut (Huber *et al.*, 1991; Vaughan *et al.*, 1994) and establishment of the oocysts. The fact that there was no difference in infection levels between the control group and females exposed to permethrin at 48 and 72 hours indicates that, once oocysts have formed, they are not influenced by the insecticide. The explanation of the equivocal result at 24 hours might be that a proportion of ookinetes are already in the process of forming oocysts, whilst others are yet to cross the peritrophic membrane or the gut wall. This possible explanation seems likely from the results presented by Vaughan *et al.* (1994), which show a broad peak of ookinete density between 12 - 24 hours after the infective feed of *P. yoelii*

yoelii in the gut of *An. dirus* and *An. albimanus*. Sieber *et al.* (1991) also state that ookinetes of *P. gallinaceum* in *Ae. aegypti* are actively crossing the gut wall just before and after this period. Unfortunately, although this critical cut off threshold for permethrin activity on parasite development has been established as pre 48 hours, or prior to oocyst formation, it does not indicate exactly which mechanism is responsible. It may even be that more than one process is inhibited during this period. The fundamental problem of pinpointing the exact stage of development arrested in this way is that permethrin, or at least its physical or biochemical effects on the insect, will extend some time beyond the time of exposure. In this way treatment at 1 hour, when exflagellation and zygote formation occurs, may not affect this process but a much later one, say ookinete migration. It is clear therefore, that any attempt to elucidate this phenomenon further will need to investigate very specific biochemical or developmental features of the parasite or host / parasite relationship.

CHAPTER 10 Infection studies using other insecticide groups

10.1. Introduction and aims of this chapter

A number of comparative infection studies were designed, substituting the pyrethroid insecticide treatments with a representative range of other insecticide groups. In each case a mosquito colony was selected with a known resistance status to a specific insecticide, and a preliminary investigation was performed to ensure the females of that strain were susceptible to infection with *P. yoelii nigeriensis*. The aim of this study was to determine whether the phenomenon of reduced prevalence of infection following exposure to pyrethroids also occurs with other insecticide classes.

10.2. Materials

10.2.1. Mosquitoes

ZANDS	*Anopheles gambiae*
DUB/APR	*Anopheles stephensi*
ST.MAL	*Anopheles stephensi*
FEST	*Anopheles albimanus*

10.2.2. Parasites

N67	*Plasmodium yoelii nigeriensis*

10.2.3. Insecticides

Malathion	5% on paper	(standard W.H.O. discriminating dose)
Propoxur	0.1% on paper	(standard W.H.O. discriminating dose)
DDT	4% on paper	(standard W.H.O. discriminating dose)

10.3. Methods

10.3.1. Effects of an organophosphate

Groups of 50 female *Anopheles stephensi* ST.MAL malathion-resistant strain were provided with an infective feed then, after one hour, the treatment group was exposed to papers impregnated with 5% malathion for 2 hours. The control group was exposed to an olive oil control paper for a similar period, and all females were then held for 10 days prior to dissection.

10.3.2. Effects of a carbamate

Random groups of 50 *Anopheles albimanus* females from the propoxur-resistant strain FEST were offered an infective feed one hour prior to treatment. One group was exposed to 0.1 % propoxur for 2 hours, the second, control group, to an olive oil control paper. Once again, all survivors were held for 10 days before infection rates were determined.

10.3.3. Effects of an organochlorine

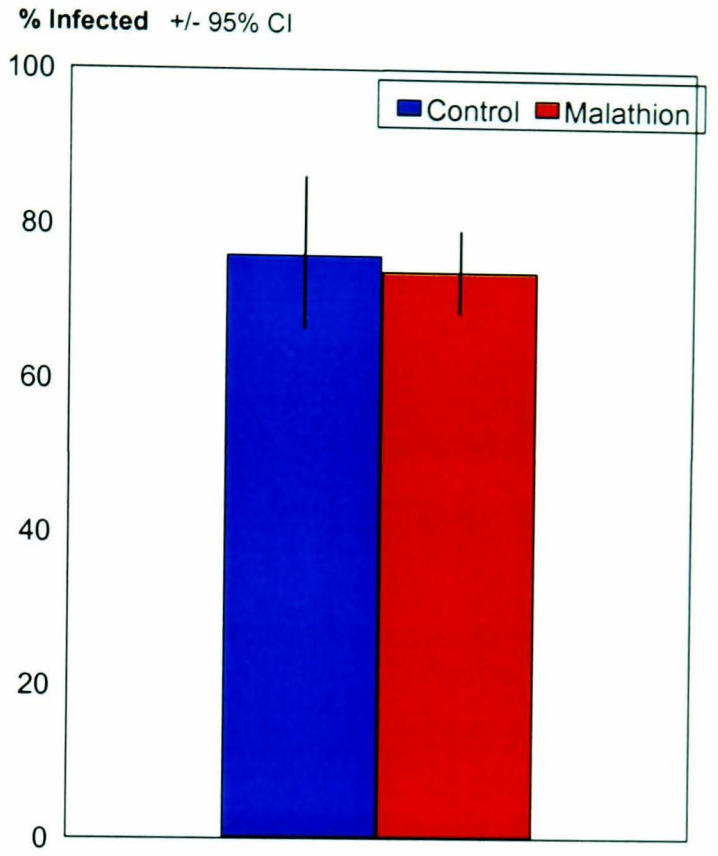
An exposure to 4 % DDT or an oil control for 3 hours was given one hour after the infective feed. The surviving females of the DDT-resistant *Anopheles gambiae* strain ZANDS were then held for 10 days before determination of infection and the recording of oocyst numbers.

10.4. Results

10.4.1. Organophosphate

Figure 30 shows that exposure to the organophosphorus insecticide, malathion, has no inhibitory effect on malarial development in the mosquito. Mean treatment mortality in exposed group was 61 %.

Figure 30. **Effect of malathion exposure on infection rates of *Plasmodium yoelii nigesriensis* in *Anopheles stephensi***



This conclusion is verified by applying a Mantel-Haenszel Chi square test in a stratified manner to allow for variation between 4 replicates (see chapter 7).

Table 24. **Effect of the organophosphate malathion on infection.**

Replicate	Control	Treated
1	14 / 20	16 / 20
2	12 / 20	11 / 20
3	12 / 20	10 / 16
4	16 / 22	15 / 21

$$\chi^2_{MH} = 0.00 \ (df=1) \qquad p = 0.978$$

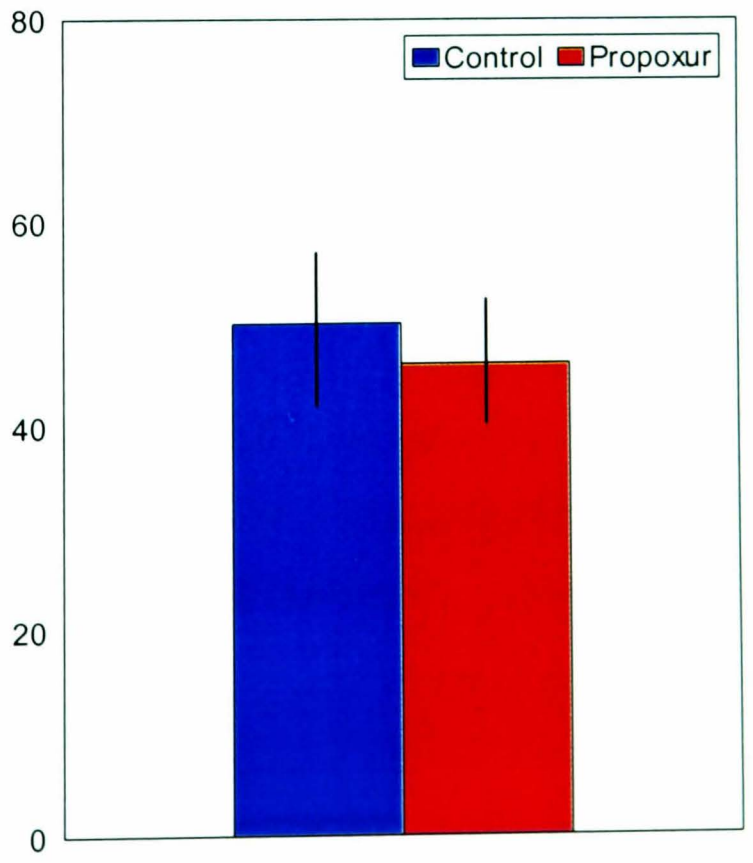
$$RR = 0.98 \quad (0.79 - 1.22)$$

Indicating no significant difference in the proportion of females becoming infected following exposure to malathion. Mean infection prevalence in treated individuals were similar to that in the controls, 74.2 % ± 5.9 % and 76.1 % ± 9.2 %.

10.4.2. Carbamate

It is clear from figure 31 that infection rates are unaffected by treatment with the carbamate insecticide, propoxur. Mean 24 hour mortality in the treatment group was 48 %.

Figure 31. **Effect of propoxur exposure on infection rates of *Plasmodium yoelii nigeriensis* in *Anopheles albimanus.***



Once more, a Mantel-Haenszel Chi Square confirmed that there was no significant difference in infection levels between the carbamate treated and control groups over 4 replicates.

Table 25. Effect of the carbamate propoxur on infection.

Replicate	Control	Treated
1	9 / 18	9 / 20
2	10 / 25	9 / 21
3	8 / 20	7 / 20
4	12 / 25	11 / 23

$\chi^2_{MH} = 0.00$ (df=1) $p = 0.959$

RR = 1.04 (0.74 – 1.45)

Indicating no significant difference in the proportion of females becoming infected following exposure to propoxur. Mean infection prevalence in treated individuals were similar to that in the controls, 46.3 % ± 7.3 % and 50.4 % ± 8.8 %, respectively (RR= 1.04).

10.4.3. Organochlorine

The results of DDT treatment on infected mosquitoes shown in figure 32 and analysis by stratified Mantel-Haenszel Chi Square test (4 replicates) confirmed that no inhibition of parasite development was induced by this compound. Mean 24 hour mortality in the treatment group was 52 %.

Table 26. **Effect of the organochlorine DDT on infection.**

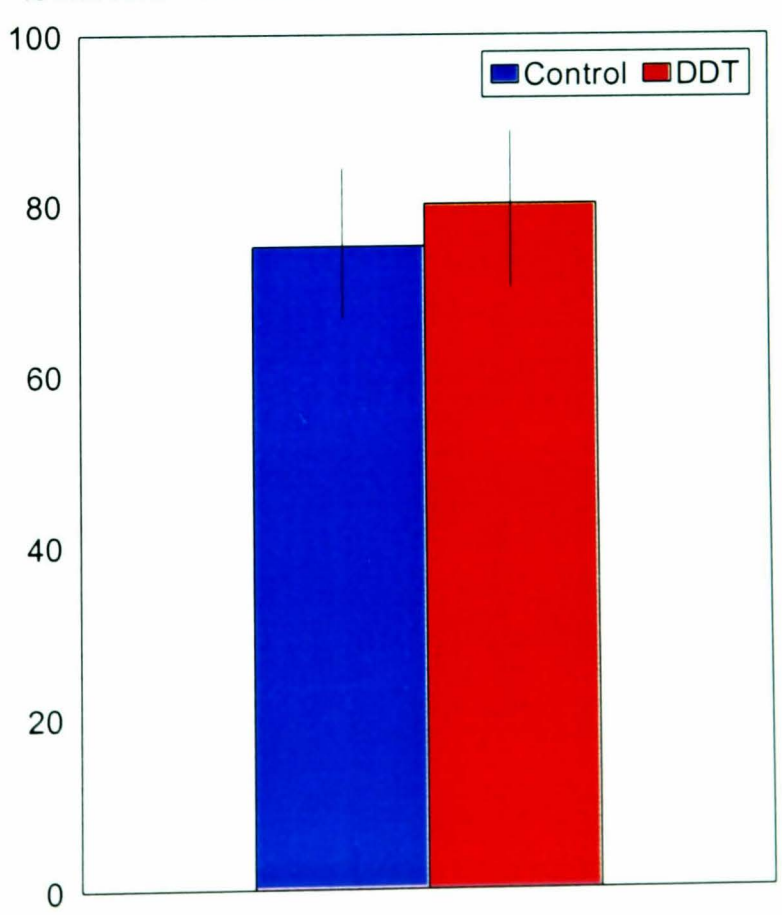
Replicate	Control	Treated
1	15 / 20	16 / 20
2	10 / 15	8 / 14
3	17 / 25	14 / 23
4	14 / 20	12 / 16

χ^2_{MH} = 0.00 (df=1) p = 0.972

RR = 1.02 (0.83 – 1.26)

Indicating no significant difference in the proportion of females becoming infected following exposure to DDT. Mean infection prevalence in treated individuals were similar to that in the controls, 80.2 % ± 8.1 % and 75.1 % ± 7.9 %, respectively (RR= 1.02).

Figure 32. **Effect of DDT exposure on infection rates of *Plasmodium yoelii nigesriensis* in *Anopheles gambiae.***



10.5. Discussion

In all three different insecticide classes investigated, organochlorine, carbamate and organophosphorus, there were no effects, detrimental or otherwise, seen on malaria infection rates. This corroborates the findings of several other groups ; Prasittsuk & Curtis (1982), with DDT and malathion ; Mahon (1955) with DDT ; Rifaat *et al.* (1974) with DDT and temephos. On this basis, it seems fair to assume that, since parasite inhibition has been demonstrated using at least 3 different pyrethroid compounds, this phenomenon is a feature specific to this class of insecticides. Thus it appears to be a unique side effect of using such compounds for the control of malaria vectors, particularly in situations such as bednet impregnation. When pyrethroids are used in such a way there is the highest probability that some contact will be made by the insect with the pesticide whilst an infective feed takes place. Such a combination of sublethal contact, should it occur, during take up of malarial parasites could be expected to result in a lower than normal prevalence of infection in that individual. Many other factors influence the choice of insecticide which are suitable for bednet use, such as mammalian toxicity, speed of kill and persistence. It is fortunate that the ideal candidates on all these counts are the pyrethroids, rather than one of the other classes of insecticide which do not exhibit this suppression of parasite development in the vector.

CHAPTER 11 The fate of Permethrin in the mosquito

11.1. Introduction

In an attempt to ascertain how sublethal doses of pyrethroids can influence the development or severity of infection within the mosquito host, it is important to establish likely sites of action. It is likely that these compounds exert their effects on parasite development in one of two ways; there may be an indirect effect which disrupts normal vector / parasite interactions, such as that described by Garcia *et al.* (1991), where the environmental conditions in the gut of *Rhodnius prolixus* became unfavourable to *Trpanosoma cruzi* development due to disruption of host hormone regulation. Alternatively, there may be a more direct effect of toxicity on the parasite itself, as predicted by Carle *et al.* (1986). In the latter case it is necessary to pinpoint those sites and times when there could be close insecticide / parasite proximity, at such intensity as to have any possibility of direct toxic action. From work presented in chapter 9, it can be concluded that once the parasite is established as early oocysts within the gut wall the pyrethroids no longer exert an effect. It seems most likely therefore that any direct action of the chemicals on the parasite occurs relatively quickly after uptake of gametocytes from an infective feed. To enable this to happen the insecticide must therefore encounter the parasite whilst it is still suspended in the bloodmeal or immediately after ookinetes begin to infiltrate the gut wall.

The fate of insecticides within the integument of insects following application to the cuticle is complex and varies greatly depending upon the compound used, metabolism within the insect, presence of specific detoxifying enzymes in some resistant strains, as well as initial penetration through the cuticle (Gerolt, 1983).

11.1.1. Aims of this chapter

It seems possible that pyrethroids exert their effect on oocyst development by direct toxicity on the parasite at doses which would not kill the mosquito. For this to occur, the substance must come into close contact with the organism while inside the insect gut. This study aimed to investigate whether such close contact would occur. Females were made to feed on a host through netting impregnated with radio-labeled permethrin. After feeding, the gut contents were separated and radio-activity recorded by a scintillation counter to determine if, and where, permethrin had accumulated within the gut. Findings were interpreted in relation to the likely proximity of insecticide and parasite, and the possible interactions that may occur.

11.2. Materials

11.2.1. Mosquitoes

DUB/APR *Anopheles stephensi*

11.2.2. Insecticides

Permethrin	EC incorporating ^{14}C @ 250 mg permethrin / m^2 on cotton netting. Supplied by ICI Agrochemicals Division, Fernhurst, Surrey, UK

11.2.3. Chemicals

Trichloroacetic acid	Sigma Chemical Co. Ltd, UK. Ref T4885
Triton X-100	Sigma Chemical Co. Ltd, UK. Ref. X100
Scintillation fluid	Optifluor®, Amersham International, UK

11.2.4. Equipment

Scintillation counter	Perkin-Elmer 3000 series

11.3. Methods

11.3.1. Blood feeds through a radio-labeled permethrin net

A square of netting 25cm x 25cm was impregnated at a dose of 250 mg/m², in the usual way, utilising an EC formulation of permethrin which incorporated a ^{14}C atom within its molecule. Batches of 20 hungry 3 day old "DUB/APR" females were allowed to feed through the radiolabelled permethrin net, until repletion, on an anaesthetised mouse. The treated net was stored at +4°C. Radio-labeled permethrin is not commercially available and the sample used in this study had been produced as a single batch for another, unconnected, research project. Access to the sample was strictly limited, and for that reason, only 3 replicates could be conducted using a total of 60 mosquitoes.

11.3.2. Blood meal collection

One hour after feeding in this manner the engorged females were knocked down with chloroform and placed on Whatman's No. 1 filter paper. As a control, a normal permethrin net was substituted for the radio-labeled net. The tips of the abdomen were snipped with a scalpel and the bloodmeal was gently squeezed onto the filter paper using a plastic spatula, making sure no cuticle or extraneous tissue contaminated the sample. Up to 20 blood spots were collected on each filter paper which were stored dry for between 14 and 17 days at + 4 °C prior to being analysed.

11.3.3. Analysis of ^{14}C permethrin in blood meals

A modification of the method described by Baer *et al.* (1989) for the preparation of blood samples to investigate adenosine transport in erythrocytes was developed as follows:

10 blood spots were eluted in 2ml PBS for 1 hour at room temperature, with the supernatant then added to 3ml of 1%, 0.1 M Triton X buffer which lysed the blood cells. The protein was then precipitated by the addition of 3ml 10% trichloroacetic acid and,

following transfer into Eppendorf tubes, the samples were centrifuged for 5 minutes at 13,000 rpm. Aliquots of 1 ml of supernatant were transferred to scintillation vials and 4 ml of scintillation fluid added to each, prior to counting. The pellets were resuspended in PBS and pooled into one vial along with 4 ml of scintillant. All samples were counted in a scintillation counter utilising quench correction and calibrated to read ^{14}C. Counts in disintegrations per minute (DPM) were recorded and compared with both control blood spots and background radiation levels. The pellet from each replicate was placed into its own single tube for counting but, due to the higher volume recovered, the elluent was divided between 4 tubes per replicate (see table 26).

11.4. Results

Table 26. Identification of radio-labeled permethrin in blood meals

Tube	Sample	DPM Replicate 1	2	3	Mean DPM (S.D.)
1	Supernatant Control	22.36	24.81	21.09	
2	" "	23.96	18.51	20.56	
3	" "	16.38	21.08	19.61	20.77 (2.34)
4	" "	18.99	21.89	21.04	
5	Supernatant Treated	22.39	18.78	19.94	
6	" "	20.02	22.86	20.09	
7	" "	18.67	19.77	21.06	20.57 (1.82)
8	" "	20.00	18.43	24.27	
9	Pellet Control	22.21	19.72	20.86	20.93 (1.25)
10	Pellet Treated	56.36	62.37	59.97	59.57 (3.03)

There was no appreciable difference in activity between tubes 1- 4 (control elluent) and tubes 5 - 8 (treated elluent), with mean DPM values of 20.77 and 20.57, respectively. Activity in control pellet tubes showed background activity (20.93 DPM) similar to that in untreated control tubes 1 – 4 (20.77). Significantly increased activity (59.57 DPM) was seen in tube 10 which contained treated pellets, which was 2.8 times higher than

background, indicating the presence of radio-labeled permethrin (or its metabolites) in this sample.

11.5. Discussion

Utilising the radio-labeled compound it is clear that at least some permethrin does reach the mosquito gut and can be detected within the blood meal. A significant increase in disintegrations per minute was detected from the pellet sample only (tube 10), not from any of the supernatants. As the blood meals were deliberately lysed with triton-X buffer prior to counting, it is clear that no permethrin was present in the fluid of cells or the serum component. This means that the permethrin must have been bound to solid material spun down during centrifugation or within the precipitated protein material. It is known that pyrethroids often associate within blood or haemolymph proteins and particularly fatty tissue in vertebrates (Tessier, 1982; Gerolt, 1983). It is reasonable to assume therefore, that the insecticide in this instance has been spun down with fat-rich debris such as fractionated cell walls or intracellular membranes. Whatever the process which allows a pyrethroid entry to the blood meal, it is clear that once in close proximity to both developing parasite and the gut wall environment, the insecticide can influence subsequent infection rates. Where suitable facilities exist, one additional avenue of research in this area may be to conduct autoradiographs of sectioned mosquitoes having been in contact with radio-labeled pyrethroids. Such an experiment may help to determine exactly where such insecticides are deposited within the insect tissues.

CHAPTER 12 Effect of insecticides on malaria parasites

12.1. Introduction

One of the possible ways in which sublethal doses of pyrethroids may exert an influence upon *Plasmodium* infection in mosquitoes is that of direct toxicity to the parasite. In such a case the insecticide must have entered the body of the vector and then come into contact with the parasite for a long enough time, and at a sufficient concentration, to elicit a response. It has been established in this study (chapter 11) that permethrin does indeed enter the bloodmeal held in the mosquito gut following a feed through an impregnated net. Although the actual amount within that part of the insect is extremely small it cannot be dismissed as ineffective, particularly if it is somehow concentrated in vital tissues within a region which heightens parasite / insecticide interaction. In the first reports of sublethal pyrethroid exposure resulting in reduced development of the malaria infection, that by Carle *et al.* (1986), it was claimed by the authors that a direct effect on the parasite was the explanation. They included in their study a rudimentary antimalarial *in vitro* test of deltamethrin on *Plasmodium falciparum*, which indicated a reduced parasitemic index of red blood cells in such cultures. Although the evidence for this mode of action was far from conclusive, the authors believed direct toxicity to be the only explanation. As already stated, in the case of inhibition of *Trypanosoma cruzi* development in the gut of *Rhodnius prolixus*, Garcia *et al.* (1991), found that a single dose of azadirachtin was sufficient to prevent infection permanently despite repeated inoculations. This, combined with the findings reported soon after by Garcia & Azambuja (1991) that the same compound mixed with infected blood does not directly prevent the parasite's development *in vitro*, makes it certain that it is not direct toxicity, but impaired vector / parasite interaction which is the explanation. Since, in both instances reported above, *in vitro* testing of direct toxicity had great influence on the conclusions drawn it was felt desirable to conduct similar studies here. To enable a direct comparison with the deltamethrin

study detailed by Carle *et al.* (1986), a similar experimental design was chosen, although using a more rigorous and established methodology and a wider range of test samples.

12.1.1. Aims of this chapter

This aim of this study was to determine the possible direct anti-malarial activity of pyrethroids and other insecticides on cultured *P. falciparum* using a standard drug screening assay. If the pyrethroids were found to have a strong anti-gametocyte activity this would have been interpreted as an important factor in the observed sublethal effect on oocyst development. Including a representative selection of non-pyrethroid insecticides in the assay allowed a comparison of effects of compounds which have already been identified earlier as either having, or not having, a sublethal effect on infection.

12.2. Materials

12.2.1. Parasites

T9-96	*Plasmodium falciparum*	Chloroquine-sensitive reference strain

12.2.2. Insecticides

Permethrin	94.1 % Technical
Deltamethrin	99 % Technical
Lambda-cyhalothrin	88.6 % Technical
Malathion	96 % Technical
DDT	Pure compound

12.2.3. Chemicals

[^{3}H]Hypoxanthine	Activity of 43.3 mCi/mg, from Amersham International, Bucks, UK
RPMI	Serum free, glucose enriched RPMI-1640 culture medium. Product R6504. Supplied by Sigma Ltd. UK

12.3. Methods

The method employed was a variation on that described by Ekong *et al.* (1990), for the routine screening of prospective antimalarial drugs *in vitro* as follows :

12.3.1. Preparation of insecticides

1.5 mg technical grade of each of the 5 insecticides was dissolved in 50 µl absolute ethanol then further diluted with RPMI to give a final concentration in each case of 1 mg / ml. This constituted the highest dose tested, with a range of serial dilutions made from it. In each case the test samples contained less than 0.1% ethanol, a concentration which is known not to inhibit parasite growth. Initial dilution of insecticides in alcohol was required as some were not directly soluble in RPMI.

12.3.2. Specific activity of tritiated hypoxanthine

[^{3}H] Hypoxanthine was purchased lyophilised with an activity of 4.3 mCi / mg, and was dissolved in serum-free RPMI to give a final working activity of of 40 μCi / ml, then stored at -20°C in 1 ml aliquots.

12.3.3. Parasite cultivation

T9-96, a chloroquine-sensitive clone of *Plasmodium falciparum* was cultured in human A+ erythrocytes suspended in RPMI 1640 supplemented with D-glucose and 10 % human A$^+$ serum, as described by Trager & Jensen (1976) and later adapted by Fairlamb *et al.* (1985). Continuous culture was at 37°C and only vigorously growing cultures with a predominance of young ring forms were used in the inhibition tests.

12.3.4. Inhibition testing

Determination of parasite inhibition by the insecticides was carried out using the microtitre plate technique developed by Dejardins *et al.* (1979). Each insecticide was tested at 12 concentrations in 4 fold serial dilutions, with a total of 4 replicates of each. Parasite cultures with a haematocrit of 2.5 and parasitaemia of 1% were incubated in the microtitre plate wells at each concentration of the insecticides for 24 hours before adding 0.02 μCi ^{3}[H] hypoxanthine per well. Incubation continued for a further 24 hours, after which the cells were harvested using a Skatron semi-automatic cell harvester. Each sample of cells was placed, along with its filter, in 2.5 ml scintillation fluid, and these were then processed for tritium activity in a scintillation counter. Two controls were included on the plates, one containing infected red blood cells with no added insecticides, which represented zero inhibition of parasite growth, the other was uninfected red blood cells corresponding to 100 % inhibition. With tritium activity levels known for these two points it was possible to analyse the sample data converting counts per minute into % inhibition. These figures, when plotted against insecticide concentration, enabled regression analysis to derive the concentration of each insecticide at which inhibition of the parasite growth represented 50 %, the IC_{50}.

12.4. Results

Table 28. Anti-malarial activity of different insecticides

Insecticide	IC_{50}
Lambda-cyhalothrin	653.4 μg / ml
Deltamethrin	No Activity #
Permethrin	207.8 μg / ml
DDT	No Activity #
Malathion	570.1 μg / ml
Chloroquine *	0.005 μg / ml

* Example to indicate typical inhibition expected from a true antimalarial substance.

Value above micromolar range

It is clear that none of the compounds tested, with the exception of the positive control, chloroquine, demonstrated any biologically significant anti-malaria activity in this parasite inhibition assay. Both DDT and deltamethrin exhibited no detectable activity whilst the others, including permethrin and lambda-cyhalothrin, only did so at concentrations far in excess of anything which could be achievable within the insect gut as a result of external contact with treated surfaces.

12.5. Discussion

Having established that pyrethroids, albeit at a relatively low level, are present in the blood meal it seemed likely that inhibition of infection is caused by direct toxicity to the parasite. Such an hypothesis was put forward by Carle *et al.* (1986) who presented evidence of a reduced parasitemic index in red cells *in vitro* when deltamethrin was added

to cultures. Unfortunately this earlier work used a fairly crude methodology with limited comparative or control replicates included. In addition, the top dose used to achieve a modest 53% inhibition was 0.75 mg/ml of a 25 g/l insecticide solution, far in excess of anything one could expect to accumulate within an insect host as demonstrated by various penetration studies reviewed by Gerolt (1983).

Utilising a far more precise and established methodology it is clear that deltamethrin does not exhibit any measurable antiplasmodic activity, and permethrin and lambda-cyhalothrin only do so at unrealistically high doses. Indeed, if a direct toxicity to malarial parasites was the explanation for infection inhibition in the vector, one might also expect the organophosphate, malathion, to inhibit *Plasmodium* in the insect, as do pyrethroids. This is because it has a higher antimalarial activity than both lambda-cyhalothrin and deltamethrin in this assay, and it is known to be very lipophilic and easily crosses insect cuticle (Gerolt, 1983). However, we have already established that malathion does not inhibit infection (chapter 10), which makes direct toxicity even less likely to be the mechanism behind this phenomenon.

It is important to emphasise that this inhibition assay was conducted on a different stage of the life cycle to the one encountered within the vector gut. Unfortunately, similar studies on zygotes or ookinetes are not yet possible due to problems with isolation or *in vitro* culturing techniques. Until this difficulty is overcome it is not possible to exclude the possibility that direct toxicity of pyrethroids on the stages of the parasite which occur in the insect is the underlying cause of their ability to inhibit development within the host.

Chapter 13 Effects on exflagellation

13.1. Introduction

13.1.1. The process of gametogenesis and exflagellation

Having proved that the mechanism by which pyrethroids inhibit development of malarial parasites in mosquitoes occurs within the first hours following intake of an infected blood meal (chapter 9), it was clear that the early developmental stages during the establishment of the parasite should be studied further. The earliest change which occurs following an infected feed is gametogenesis and exflagellation, triggered by both environmental and host mediated factors. Even by simply exposing a drop of infected blood to ambient laboratory air, Nijhout & Carter (1978) observed the transformation of elongated *P. gallinaceum* gametocytes into spherical cells. The cytoplasm of the host cell became translucent and the membrane stretched out to form filamentous vesicles prior to the disappearance of all host cell material. This process is known as emergence and takes around 6 - 8 minutes to complete (Wernsdorfer & McGregor, 1988). Following emergence, the male gametes undergo further transformation forming protrusions of nucleated motile flagellae of 20 - 25μ length (Bruce-Chwatt, 1980) from their surface. The vigorous motion attracts nearby cells, including female gametes, to form clumps and the female gametes are subsequently fertilised as the male microgametes detach and swim free. Carter *et al.* (1979) observed exflagellation *in vitro* and stated that the whole process from gametogenesis to fertilisation takes 10 - 20 minutes before the zygote is formed. As the process of gametogenesis, emergence and exflagellation occur concurrently (Wernsdorfer & McGregor, 1988) they will be referred to under the general term 'exflagellation'.

13.1.2. Environmental regulation of exflagellation

There are several environmental factors which can affect exflagellation, the main ones being ;

i) Temperature. Under normal conditions the temperature of the vector midgut environment is around 20 - 26°C, and times quoted in this thesis for specific events during development of the parasite are expressed assuming temperatures within this range. At temperatures between 28 and 36°C, Roller & Desser (1973) have reported a much higher rate of development, although the process is arrested in *P. falciparum* at 38°C and above (Sinden & Smalley, 1976). Reducing the temperature to between 14 - 16°C increases the time for exflagellation by around 10 times, and below 10°C the process does not proceed (Wernsdorfer & McGregor, 1988).

ii) Blood pH. Although some research groups quote results of changes in terms of gas tension of CO_2 in the blood meal (Bishop & McConnachie, 1956) it is really the effect this has on pH that is important in exflagellation. This was best demonstrated in experiments conducted by Nijhout & Carter (1978) who studied exflagellation in *P. gallinaceum* within airtight chambers with regulated gas tension and ionic composition of the medium. During these studies it was found that exflagellation was initiated only within the very narrow pH range of 7.8 to 8.4. The most interesting finding was that it is only the initial triggering of exflagellation which requires this optimal pH for just 10 seconds. Once underway, the bicarbonate buffer could be removed and the process continued to completion even when the pH fell to 7.4. Sinden and Kawamoto (1991), concluded that two independent ionic exchange mechanisms are responsible for initiating changes during microgametogenesis which result in the export of H^+ ions.

13.1.3. Insect mediated factors affecting exflagellation

It is clear that there are certain substances within the mosquito itself which can play an important part in the initiation of exflagellation. Such substances, designated mosquito exflagellation factor (MEF) by Nijhout (1979), can act in one of several ways.

Gametocytes of *P. elongatum* do not spontaneously exflagellate in favourable environmental conditions *in vitro,* or even when taken up into hindguts of aedine or anopheline mosquitoes. However, when imbibed by *Culex quinquefasciatus* or *Cx. pipiens*, Micks *et al.* (1948) reported rapid and vigorous exflagellation. A particularly interesting feature of this parasite species was also described by the same group, whereby the addition of gut extracts from *An. quadrimaculatus* or *Ae. aegypti* actually retarded this process in the normal *Culex* vector. Such findings therefore suggest a vector specific factor which inhibits exflagellation, and may even go some way to account for development of infection in only some, but not other, mosquito species. The importance of initiation of exflagellation as a regulatory factor in determining vector competence within anopheline species is, however, considered unlikely by Mendis *et al.* (1994). They found little or no variation in *P. falciparum* exflagellation response in the midguts of 6 different anopheline species. With the notable exception of *P. elongatum* described above, most malarial parasites exflagellate to some extent on contact with air, without insect contact. However, it is clear that MEF's do play a significant role in natural vector / parasite interactions during establishment of an infection. Eyles (1952) showed that washed *P. gallinaceum* gametocytes could be forced to exflagellate within mosquitoes even if fed in a salt solution instead of whole blood. Such an experiment, later repeated by Nijhout (1979), shows that a normally inhibitory event, such as removal of blood and plasma from gametocytes, can be overcome by the presence of MEF. Attempts to characterise MEF by the same author demonstrated this factor in *An. stephensi* anal excretions, the midguts of unfed *Ae. aegypti* of both sex and extracts of crushed mosquito heads. It was reported as a heat stable peptide of around 10 KDa by Sinden & Kawamoto (1991). In recent years, work conducted in this field has yielded little understanding of this most fundamental step in the malaria life cycle. Not until Garcia *et al.* (1997) characterised a substance purified chromatographically from the gut, head and lumen of anopheline mosquitoes was progress made. This group revealed a heat-stable, hydrophilic molecule as a negatively charged

chromophore. By this time, groups working on MEF had renamed the substance Gametocyte Activating Factor or GAF (Billingsley & Sinden, 1997; Billker *et al.*, 1998). Without doubt, the most promising discovery made in this area in recent years, as pointed out by Carter & Ranford-Cartwright (1998), is that published by Billker *et al.* (1998) who have identified the GAF / MEF molecule as xanthurenic acid. This chemical, a by-product of insect tryptophane metabolism within the kynurenine pathway, was isolated and found to cause high exflagellation rates *in vitro,* whereas no such activity was found using several structurally similar molecules. The substance was also observed to dramatically widen the pH range over which exflagellation would occur. Xanthurenic acid is known to be linked to eye colour pigmentation in insects, a pathway which has already been elucidated and disrupted in *Drosophila* mutation studies. It is not clear how these two processes of exflagellation and eye pigmentation may be associated, if indeed they are, but it would be interesting to look into this further, perhaps with the aid of anopheline strains with eye-colour mutations. It is clear that having identified the "key" to exflagellation, the first real rate-limiting step in the parasite's life cycle within the vector, this has opened up a new avenue for transmission blocking interventions. If GAF does turn out to be as important to parasite development as some think it is, it may be possible to target this process in vector mosquitoes by engineering mutants unable to synthesise xanthurenic acid. What is clear is that final vector capacity of the mosquito will be affected by the efficacy of each stage of the parasite's development within it (Billingsley & Sinden, 1997), so any disruption to this process may well have an influence on malaria transmission.

13.1.4. Aims of this chapter

Exflagellation is one of the earliest processes in establishing an infection, occurring soon after the infective meal is imbibed. As several factors have been shown to influence the initiation and rate of exflagellation, this was considered as one possible target which could

be affected by pyrethroid exposure. Exflagellation can be readily observed *in vitro* and this study aimed to determine the effect of permethrin on this process, with the findings discussed in terms of how this may influence subsequent infection rates.

13.2. Materials

13.2.1. Parasites

N67 *Plasmodium yoelii nigeriensis*

13.2.2. Insecticides

Permethrin 94.1% technical grade

13.3. Methods

13.3.1. Induction and scoring of exflagellation *in vitro*

The technique described by various workers (Vaughan *et al.*,1994; Mendis *et al.*, 1994) for studying exflagellation kinetics *in vitro* was adapted as follows :

Wet mounts of blood from *P. yoelii nigeriensis* gametocytaemic mice were collected by tail snip. The spots of approximately 4 μl were incubated at room temperature on glass slides and covered with a glass coverslip. Observations were made every 2 minutes during the period 5 - 15 minutes after collection. The number of exflagellating centres per 20 fields were recorded in a total of 8 replicates, using a phase contrast microscope at 400x.

13.3.2. Inhibition of exflagellation by pyrethroids

Gametocytaemic blood was again collected as described above but on this occasion was added to 5 μl of 1 % permethrin in PBS or PBS control, then gently mixed with a micropipette. PBS and PBS / permethrin solution were checked to ensure an identical pH to remove this possible confounding variable. Having established that exflagellation reached a peak at 10 minutes using the techniques detailed above, counting took place after

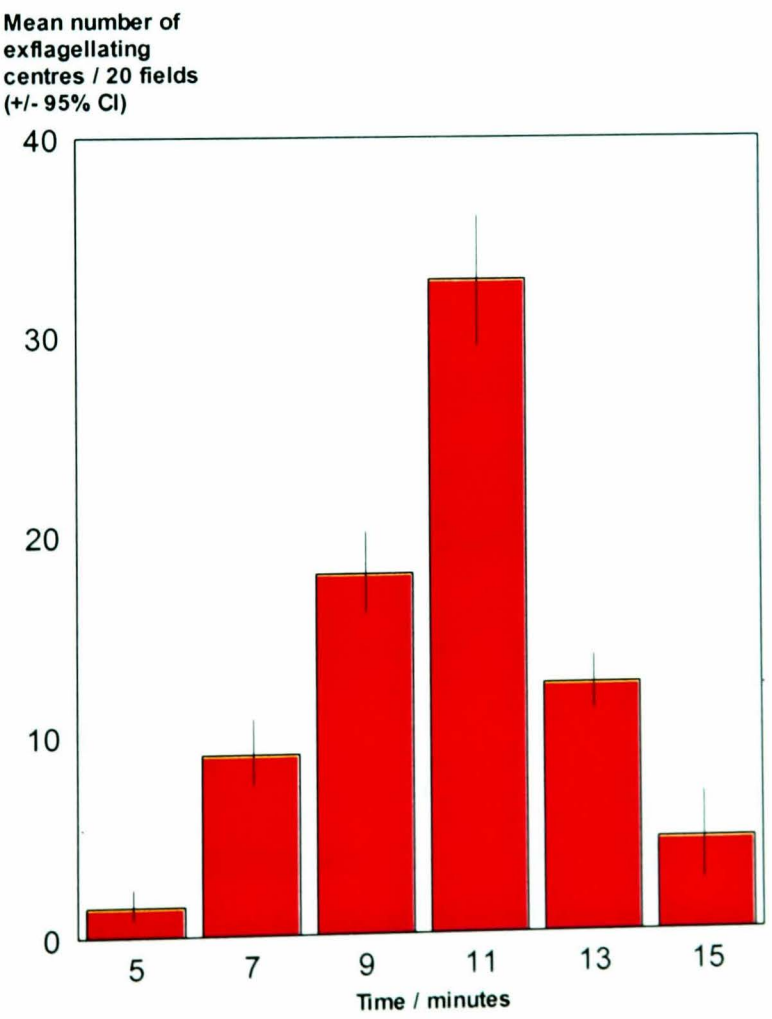
this period of incubation in each case. The number of exflagellating bodies per 20 fields were recorded as before. A total of 8 replicates were conducted for both treatment and control groups using blood collected from the same infected mouse, thus minimising inter-mouse variability of gametocytes.

13.4. Results

13.4.1. Exflagellation kinetics

The total number of exflagellating centres (20 fields x 8 replicates) recorded at 2 minute intervals over the period 5 - 15 minutes after exposure to air incubation is shown in figure 33. It is seen that the highest rate of exflagellation occurs at 9 - 10 minutes, whilst prior to 7 minutes and after 13 minutes the process is at a very low level.

Figure 33. Exflagellation kinetics of *Plasmodium yoelii nigeriensis*



13.4.2. Effect of permethrin on exflagellation

Table 29. The effect of 5 μl of 1 % permethrin permethrin on exflagellation

	Number of exflagellating centres / 20 fields	
Replicate	**Control**	**Permethrin**
1	31	28
2	40	42
3	43	41
4	39	43
5	40	41
6	34	40
7	32	36
8	37	39
Total	296	310
Mean	37.00	38.75
S.E.M.	1.51	1.71

Normality (Anderson – Darling Test) $p = 0.657$

Paired t-test $t = 1.62$ $p = 0.15$ indicating that there is no significant difference in exflagellation in gametocytes with or without the presence of permethrin.

13.5. Discussion

13.5.1. Exflagellation kinetics

In vitro exflagellation of *Plasmodium yoelii nigeriensis* was observed under ambient conditions beginning at around 7 minutes and subsiding by 15 minutes. After exposure to

air the peak activity was found at 10 - 11 minutes, a figure similar to that quoted by Vaughan *et al.* (1994) in *P. yoelii yoelii* of 8 - 10 minutes. The fact that exflagellation occurred so readily under such conditions suggests that a mosquito initiation factor is not a prerequisite to initiate the process in this species, at least *in vitro*. In this respect, this parasite species differs from *P. elongatum* which will only form gametes within the gut of specific vector mosquitoes (Micks *et al.*, 1948). Even so, it may be that MEF (GAF) could play a role in enhancing the rate of exflagellation if present. Vaughan *et al.* (1994) calculated that there can be up to a 55 fold loss during the conversion of ookinetes of *P. yoelii* into oocysts. It could therefore be that the rate of spontaneous exflagellation observed *in vitro* would not be high enough to ensure an ookinete population large enough to overcome such potential losses and ensure infection. If this is true, anything which inhibits production, release or activity of MEF may have a profound effect on infection rates or parasite burden.

13.5.2. Effects of permethrin on exflagellation

No significant change in rate of exflagellation was observed in gametocytemic blood in the presence of permethrin. It is apparent therefore, that the recorded decrease in infection rates caused by this compound is not caused directly by inhibition of this process. However, the recent work by Billker *et al.* (1998) which identifies MEF (GAF) as xanthurenic acid, synthesised within the ommochrome pathway which determines eye pigmentation colour of insects, opens much wider possibilities for an indirect mode of inhibitory action. As pointed out by Carter and Ranford-Cartwright (1998), the fact that there is likely to be a low level of this chemical in mammalian blood ingested along with gametocytes during a mosquito feed, implies there is a threshold level required to initiate exflagellation. Consequently, any action by pyrethroids which has a physiological effect on an insect interfering with normal metabolism may reduce the production of this important molecule. Having established what far-reaching effects sublethal doses of these

compounds exert on insects such a possibility cannot be ruled out. If it becomes possible to detect and monitor specific production of xanthurenic acid within mosquito bodies it would be interesting to investigate this possible cause of pyrethroid induced reduction in parasite development.

CHAPTER 14 Effects on ookinete survival and penetration

14.1. Introduction

Mature gametocytes taken up from vertebrate host blood by a feeding mosquito must rapidly adapt to a very different environment within the invertebrate host. The parasite must withstand attack not only from the vertebrate antibodies and complement taken up with the blood meal, but additional insect host defences also. Once within the mosquito gut the parasite must penetrate cellular and acellular physical barriers and resist an array of digestive enzymes, macromolecules and lectins (Warburg & Miller, 1991; Shahabuddin & Kaslow, 1994). At least one mechanism of refractoriness in mosquitoes is based on the killing of ookinetes. Vernick *et al.* (1995) reported death of *Plasmodium gallinaceum* ookinetes in contact with the midgut wall of *Anopheles gambiae* prior to oocyst development at around 27 hours after the infective feed, although the mechanism is as yet unknown. Under normal conditions the number of ookinetes fed to a mosquito influences the oocyst rate. Miller (1991) reported that when 10 ookinetes of *P. gallinaceum* are fed to *Aedes aegypti* mosquitoes, around 50% establish oocysts. When, however, 10000 ookinetes were imbibed, only a small percentage survived, the majority being attached to, or trapped within, the peritrophic membrane.

14.1.1. Trypsin activity and ookinete survival

In order to utilise blood as a protein source for egg production, mosquitoes secrete a number of proteolytic enzymes to digest the meal (Gooding, 1966). In *Culex nigripalpus*, Borovsky (1986), attributed 80% of midgut proteolytic activity to trypsin, on the basis of experiments using an enzyme specific inhibitor, tosyl-*L*-lysine chloromethyl ketone. The relative importance of trypsin was demonstrated further by Borovsky & Schlein (1988), who found that a specific inhibitor of chymotrypsin only blocked 23% of the total protease activity in the same insect species. Prior to a blood meal, Gooding (1973) reported only

weak proteolytic activity in *Ae. aegypti* which rose to a peak at around 24 hours after the blood feed and subsequently declined. Briegal & Lea (1979) found that the peak of trypsin activity was under the control of endocrine factors from both the medial neurosecretory cells and the ovaries. An excellent mini-review of trypsin synthesis at the molecular level has been published by Noriega and Wells (1999). The authors conclude there are two distinct phases of trypsin production in *Ae. aegypti*; a rapid but small release of "early" trypsin between 4 – 6 hours post-feed which acts as a "sensor", which then stimulates a more substantial production of "late" trypsin which is responsible for the majority of endoproteolytic cleavage. Although it is known that both synthesis and secretion of trypsin are affected by factors originating from the physical process of blood feeding, as well as endocrine regulation, the exact process remains unclear. Perhaps not surprisingly, Cázares-Raga *et al.* (1998) found a range of sex specific proteases in *An. albimanus* fed on sugar solution using 2-dimensional electrophoresis, suggesting that at least some gut enzymes are under sex dependent regulation. When Feldmann *et al.* (1990) studied enzyme levels in *An. stephensi* strains which were either refractory or susceptible to *P. falciparum*, they found that trypsin alone played no part in determination of whether or not an infection developed. Work with *Ae. aegypti* infected with *P. gallinaceum* by a number of groups (Gass, 1977; Gass & Yeates, 1979; Yeates & Steiger, 1981) has shown that trypsin can, however, affect parasite development under different circumstances. Ookinetes are damaged during contact with purified proteinases *in vitro* and similarly, if digestion is stimulated prior to *in vivo* infection, there is destruction of immature ookinetes within the mosquito gut. In other invertebrate host / parasite interactions the parasite may even modify trypsin synthesis and activity. Dillon & Lane (1993a) demonstrated that *Leishmania major* suppresses protease activity and prolongs the digestion period in infected *Phlebotomus papatasi*. In this way they believe that the parasite not only has less chance of damage due to the enzyme, but it has a food source from the digesting bloodmeal over a prolonged period. In a similar manner, Imbuga *et al.* (1992) found

membrane preparations of *Trypanosoma brucei* inhibited trypsin activity in *Glossina morsitans*. In *Leishmania,* parasite synthesised chitinolytic enzymes play an even more specific role in transmission, by structurally damaging the cuticle lining of the feeding pump valves of its sandfly vector. On this basis, Schlein *et al.* (1992) postulate that there is an increased back-flow from gut to host tissue during feeding, which increases the number of parasites being transmitted.

The detrimental effects of pyrethroids on nervous tissue and endocrine function may result in disruption of the normal proteolytic enzyme production causing a reduction in survival or establishment of the pre-oocyst stages. This could be a result of either an increase in activity destroying the parasite directly, or a decrease in activity which would hinder the ookinete penetration of the peritrophic membrane (Shahabuddin *et al.*, 1993). To investigate whether this is the case, trypsin activity was determined in treated and control mosquitoes at a range of times before and after the bloodfeed.

14.1.2. The peritrophic membrane and ookinete migration

Following a meal, most haematophagous insects secrete a sac-like peritrophic matrix which surrounds the gut contents (Peters, 1992). In mosquitoes this consists of a fibrous, chitin-rich layer which forms around the blood meal anteriorly to posteriorly within a few hours of the feed. This peritrophic membrane (PM) is secreted by all midgut columnar cells (Clements, 1992), surrounding the blood meal within 4 hours and is discernible as a fully hardened, discrete layer by 12 hours after ingestion. The formation is assumed to be independent of the meal content as several authors have found it present following introduction of a saline or air enema (Freyvogel & Jaquet, 1965) or latex particles plus ATP (Billingsley & Rudin, 1992). The exact function of the PM is not known, it forms too late to afford protection against cellular invasion from arboviruses (Hardey *et al.*, 1983) or microfilariae (Townson & Chaithong, 1991). Ponnudurai *et al.* (1988), however, suggest

that in some *Anopheles / Plasmodium* combinations it may slow migration of the ookinete to the midgut epithelial cells to such an extent that they succumb to the digestive process. It may even be that ookinetes could fail to penetrate the PM at all once it is fully developed. Whatever the function of the PM, it is clearly an obstacle for the ookinete in its movement from the blood meal into the gut cell wall. Sieber *et al.* (1991) observed numerous ookinetes trapped in the endoperitrophic space, presumably blocked from further development. The same group also reported that those ookinetes in the process of crossing the PM had electron dense material at their apical end and that disruption of chitin fibrils was evident. Huber *et al.* (1991) later demonstrated chitinolytic activity in *in vitro* cultures of ookinetes 15 hours post fertilisation. Further investigations by Shahabuddin *et al.* (1993) used a specific chitinase inhibitor, allosamidin. This completely blocked oocyst development, confirming the role of chitinases in ookinete penetration of the PM. The same group also found that, when *in vitro* cultured ookinetes were treated with mosquito midgut extracts containing normal digestive proteases, the chitinase activity increased still further. Shahabuddin & Kaslow (1994) suggested that this phenomenon is due to the parasite chitinase being produced by the ookinete as a zymogen or pro-enzyme which is activated by protease in the mosquito midgut. Consequently, it is possible that any process which interferes with the normal release of trypsin, or other proteases, by the mosquito during blood meal digestion could simultaneously reduce the chances of an ookinete penetrating the PM and establishing an infection. In other host – parasite relationships chitinase plays a key role in migration through the peritrophic membrane too. In the sandfly vector, *Phlebotomus papatasi*, the *Leishmania* parasites undergo replication within the peritrophic membrane itself, unlike malaria parasites which must move out into the surrounding gut tissue. Schlein *et al.* (1991), have suggested that in infected flies the breakdown of PM occurs prematurely at the anterior end, and is parasite mediated, whereas the natural PM breakdown in uninfected flies is from the posterior end and under insect control. It is clear that chitinase produced by a variety of parasites plays a key role

in their development, and, as emphasised by Shahabuddin & Kaslow (1993 and 1994), could prove a useful target for blocking transmission.

14.1.3. Ookinetes and the gut wall

Once it has migrated through the peritrophic membrane, the ookinete encounters one more physical barrier before it can continue development. There is still considerable controversy as to the mechanism by which the ookinetes pass from the midgut lumen to the haemocoel side of the epithelium. Early suggestions made by Garnham *et al.* (1962) were that the parasites migrated intra-cellularly using a proteolytic substance secreted by micronemes. Subsequent observations by Canning & Sinden (1973), favoured an intra-cellular route for the species *P. berghei*. However, electronmicroscope studies by Meis & Ponnudurai (1987), and shortly after, Meis *et al.* (1989), observed intercellular ookinetes of *P. falciparum,* but intracellular ookinetes of *P. berghei,* within the midgut epithelia of the same mosquito species, *An. stephensi*. It seems likely, therefore, that the ookinetes are able to pass either inter- or intra-cellularly, but in both cases, the importance of proteolytic enzyme secretion in highly likely. A publication by Syafruddin *et al.* (1991) including a large number of highly informative electronmicrographs, clearly shows migrating ookinetes of *P. yoelii nigeriensis* moving through the midgut wall of *An. omorii* via an inter-cellular route, although some individual parasites were also seen within cells. The same group reported the appearance of vacuoles of unknown origin around migrating ookinetes, but no clear evidence of electron density which might support the theory of proteolytic secretion by parasite micronemes.

14.1.4. Aims of this chapter

Having reviewed the complex process and regulation of ookinete migration through the peritrophic membrane and penetration of the gut wall, another potential mechanism by which pyrethroids may exert their effect on infection rates has been identified. This study aimed to investigate the effects of permethrin on two of the most important regulating

enzymes in this process, trypsin and chitinase. The effect of permethrin on chitinase activity was observed using a modified biochemical assay in comparison with an untreated control and a known chitinase inhibitor. Gut trypsin activity was compared in batches of untreated or treated *An. gambiae* females at three specific time-points after feeding. Findings are discussed in relation to the possible effects of pyrethroid exposure on ookinete development and migration prior to development of oocysts.

14.2. Materials

14.2.1. Mosquitoes

DUB/APR *Anopheles stephensi*

14.2.2. Insecticides

Permethrin 0.25 % on paper

14.2.3. Chemicals

BApNA	Benzoyl-arginine-*p*-nitroanilide. Sigma ref. B-3133
DMF	Dimethylformamide. Sigma ref. D-4254
TRIS- HCL	Trizma hydrochloride base. Sigma ref. T-3253
4-Methylumbelliferone	Sigma ref. M-1381
4-Methylumbelliferyl-*N,N',N''*-triacetyl-ß-chitotriose	Sigma ref. M-5639
Chitinase	From *Streptomyces griseus*, Sigma ref. C-6137

14.2.4. Equipment

Spectrophotometer	Beckman LS 6000 LL
Microplate reader	UV Max Enzyme kinetics microtitre plate reader, utilising Soft- Max software, Molecular Devices Corp., USA

14.3. Methods

14.3.1. Protein assay

To ensure a fair comparison between the various treatment and control groups of mosquitoes, specific enzyme activities are presented as a proportion of total protein present in a sample. A standard commercial protein assay kit (Bio-Rad) based on Coomassie blue dye binding (Bradford, 1976) was used with absorbances read at 570nm. Protein assays were always run on 100μl samples from the same insect homogenate as used in the trypsin assay, and conducted within a matter of minutes of each other.

14.3.2. Trypsin assay

Trypsin assays were conducted on individual mosquitoes following the technique described in full by Dillon & Lane (1993) who did similar experiments on the sandflies *Phlebotomus papatasi* and *P. langeroni.*

Assays were performed in wells of microtitre plates on individual insects homogenised in eppendorf tubes. 2mM benzoyl-arginine-*p*-nitroanilide (to give 1mM final dilution) was prepared in 7% dimethylformamide in 50mM Tris HCL buffer at pH 8.2. An individual mosquito was homogenised using a clean plastic rod in 500μl buffer. 100μl of mosquito homogenate was added to an individual well of the plate then, when all wells were complete, 100μl of substrate was added to each well. The plate was immediately placed in the plate reader and read kinetically over 5 minutes at 405nm. Trypsin activity in the mosquito gut was calculated as specific activity / minute, then correlated with the results of the corresponding protein analysis of the individual insect to derive the specific activity / mg insect protein in μM (of substrate hydrolysed) / minute.

14.3.3. Effects of permethrin on trypsin activity

Two random groups of twenty 2 - 3 day old *An. stephensi* (DUB/APR) females were exposed to a sublethal dose of permethrin or an oil control using the standard WHO adult insecticide test kits. After 24 hours all individuals were allowed to take a blood meal on a human arm with those not completing a full feed being discarded. At set intervals post feed, females from each group had their gut removed and stored in normal saline at -20°C prior to the trypsin assay. The time intervals were 4, 16 and 24 hours, these times being chosen as representative of different functional stages of the post-feed infection processes. An increase in trypsin at 4 hours may damage the parasite during its development from gametocyte to zygote to ookinete. A high point in chitinase activity within ookinetes at around 16 hours and the greatest numbers penetrating the PM and migrating into the epithelial wall at 24 hours (Huber *et al.*, 1991) could be hindered by reduced trypsin activity in the gut.

14.3.4. Chitinase assay

The chitinase assays described by Huber *et al.* (1991) and Kurander & Robbins (1987) were adapted as follows :

4-Methylumbelliferyl-*N,N',N''*-triacetyl-ß-chitotriose was used as a substrate as it is readily cleaved by chitinase to release 4-Methylumbelliferone, a compound which fluoresces under UV light. 4-Methylumbelliferyl-*N,N',N''*-triacetyl-ß-chitotriose was used at a concentration of 125μM in 0.1 M sodium phosphate at pH 6.8. 20 ml of this solution was incubated at 26°C for 10 minutes prior to the addition of 100μl chitinase. After mixing thoroughly it was left to incubate once more at 26°C. At 5 minute intervals from the moment of first adding the enzyme, sub-samples of 500 μl were removed from the main reaction vessel and added to 3 ml 0.5 M glycine-NaOH at pH 10.5. Raising the pH in this way stopped the reaction and allowed the levels of liberated 4-Methylumbelliferone to be

measured on completion of the experiment. Sub-sampling in this way continued every five minutes for a total of 30 minutes.

Samples were read using a spectrophotometer with excitation at 350 nm and emission recorded at 440 nm.

14.3.5. Effects of permethrin on chitinase activity

In order to establish whether permethrin has a direct effect on chitinase activity a standardised chitinase assay was performed with or without the presence of the insecticide. The assay used was that described above based on Huber *et al.* (1991) but modified to monitor potential inhibition :

Allosamidin, a known chitinase inhibitor, was found by Shahabuddin *et al.* (1993) to inhibit activity when present at 5 μM in a blood meal. At a concentration of 0.1 mM, or above, it was found to completely block transmission of *P. falciparum* in *An. freeborni*. On this basis, concentrations ranging from 0.5 mM up to 10 mM permethrin were chosen to study its effect on chitinase activity in this assay. With such relatively high levels, even a small inhibitory effect should be detectable. Samples were set up as described in section 14.3.4 above, but with one set of replicates having permethrin added at either 0.5 mM or 10 mM to the solutions prior to the addition of chitinase and incubation. Sub-samples were once again removed at intervals as described in section 14.3.4 and the reaction stopped prior to reading on a spectrophotometer. As a positive control, a further sample was incubated with the known chitinase inhibitor allosamidin at 0.1 mM.

14.4. Results

14.4.1. Effects of permethrin on gut trypsin activity

Table 30. Effects of permethrin on trypsin activity

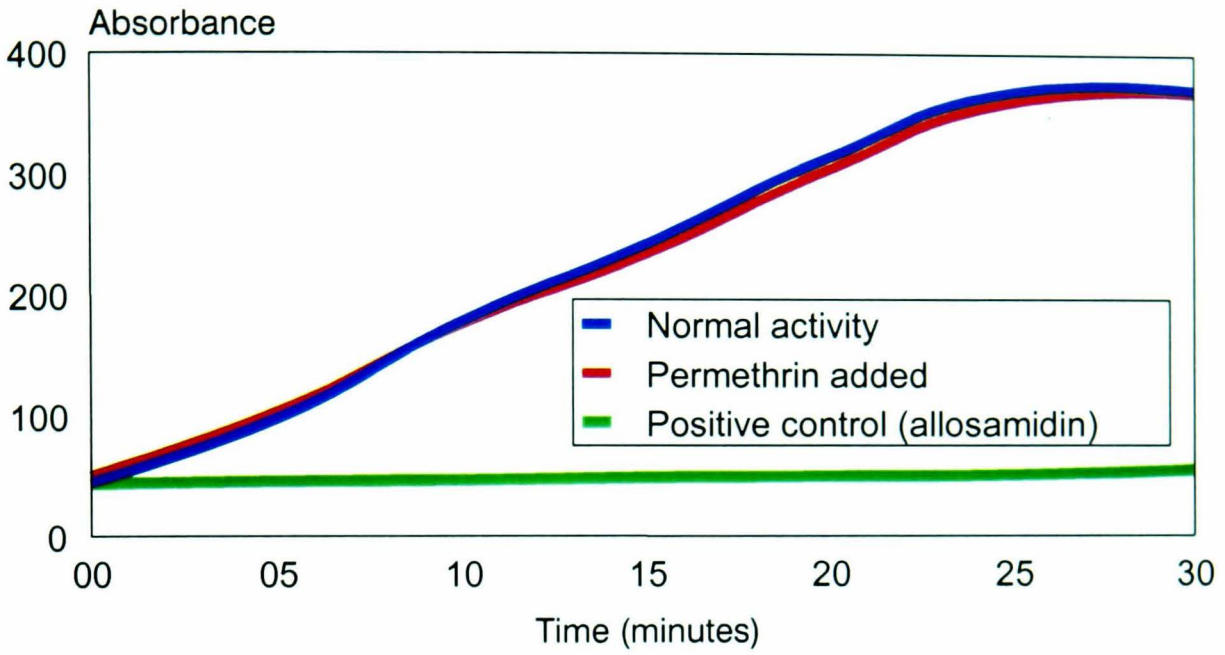
Treatment (n=20 ♀♀)	Trypsin activity	
	μM/min insect ± (SE)	μM/min mg protein^{-1} ± (SE)
Control 4 hour	8.18 ± 0.77	10.09 ± 0.95
Permethrin 4 hour	10.92 ± 1.18	12.08 ± 1.57
Control 16 hour	43.69 ± 3.32	63.10 ± 5.41
Permethrin 16 hour	55.16 ± 1.19 * (t = 3.33, p = 0.0037)	73.08 ± 4.16
Control 24 hour	26.69 ± 2.69	49.10 ± 2.91
Permethrin 24 hour	32.10 ± 2.63	33.60 ± 3.57 * (t = 3.36, p = 0.004)

* Significant difference (paired t test)

A significant increase in trypsin activity was seen in treated females at 16 hours post-exposure. However, when the total protein levels / insect were taken into account the apparent difference was found to be no longer significant. At 24 hours post-exposure there was a significant decrease ($p = 0.004$) in trypsin in treated females when expressed as a proportion of total protein.

14.4.2. Effects on chitinase activity

Figure 34. **Effect of permethrin on chitinase activity.**



14.5 Discussion

14.5.1 Effects on trypsin activity

Table 10 shows the trypsin activity in control and permethrin treated mosquito midguts at 4, 16 and 24 hours periods post blood feed. Activity is expressed in both mean activity per gut and mean activity per mg protein.

4 hours

At the earliest time point, 4 hours, there was no significant difference in enzyme activity between the permethrin treated and control groups. As expected from existing research, trypsin levels at this time are low, 8 – 11 μM / min / gut. Such activity is likely to correspond to the "early trypsin" described by Noriega & Wells (1999), who postulate this to be under translational regulation, due to its rapid appearance, stimulated by the

presence of amino acids in the blood meal and, although playing little role in the digestion process, being vital in initiating synthesis of "late trypsin". The fact that permethrin has no effect on early trypsin levels could be explained by fact that, although its synthesis is under control of juvenile hormone, transcription of the gene begins soon after adult emergence and the mRNA is stored in the midgut epithelium (Noriega & Wells, 1999). Under these circumstances, hormonal control, the most likely candidate for pyrethroid interference, has already fulfilled its role days before exposure occurs. Once amino acids in the blood meal are detected, the stored mRNA is translated rapidly and early trypsin is released.

16 hours

At 16 hours post feed, a period during which ookinetes are actively migrating through the peritrophic membrane (Huber *et al.*, 1991), we see from table 10 that there was a significant ($p = 0.0037$) increase in trypsin activity per insect in the permethrin treated group (55.16 ± 1.19 μM/min) compared with the controls (43.69 ± 3.23 μM/min). However, this only proved significant prior to being expressed in terms of activity per mg protein, a correction adopted specifically to reduce variables between batches of individual females used. Because of this it is not prudent to accept this difference as a meaningful one, as activity may have been unduly influenced by factors such as individual mosquito size or nutritional status. Even if the moderate increase in activity were to have reached significance in the treatment group, it would not have supported our earlier hypothesis that a reduction in trypsin could have resulted in lower ookinete chitinase activity, thus inhibiting their penetration of the peritrophic membrane and ultimately reducing infection levels.

24 hours

At 24 hours post feed a significant ($p = 0.004$) decrease in trypsin activity per mg protein was seen in those females exposed to permethrin. At this time point, the main process of digestion is underway, with "late" trypsin playing the major part in endoproteolytic cleavage of blood meal proteins (Noriega & Wells, 1999).

14.5.2. Effects on chitinase activity

It is clear from results presented in figure 36 that there is no noticeable effect of permethrin on chitinase activity, even at concentrations as high as 10 mM, which is far above anything which could conceivably be encountered in a mosquito blood meal, gut or surrounding tissues following the individual's exposure to external insecticide. The positive control, allosamidin, however, clearly demonstrated a dramatic inhibitory effect indicating that the method used was sufficiently sensitive to detect such an effect, had it been present. It seems highly unlikely therefore that the observed reduction in *Plasmodium* infection rates following pyrethroid contact are a result of less efficient ookinete penetration through the peritrophic membrane or midgut epithelial cells due to inhibition of chitinase activity, such as that described by Shahabuddin & Kaslow (1993 and 1994).

CHAPTER 15 General Discussion

15. Introduction

This project has investigated a wide range of effects of pyrethroid insecticides on mosquitoes. The mode of action of this class of compounds has also been summarised in an attempt to understand how and why they are biologically active, and a comprehensive review of earlier work in this field in a variety of insect species has been undertaken. Practical investigations have concentrated in three main areas, the effects of sublethal doses of pyrethroids on basic physiology, longevity and behaviour of mosquitoes and parasite transmission.

15.1. Physical effects

Investigations into the effect of sublethal doses of three of the most widely used pyrethroids in public health, permethrin, deltamethrin and lambda-cyhalothrin, have shown a marked reduction in adult longevity. The effects appear unrelated to the initial direct mortality caused by contact with the pesticides during the immediate 48 hour post-exposure period, as there is no obvious deviation between control and treatment groups during the middle period of the daily survival graphs. However, there is a significant increase in mortality towards the end of the adult life span, and this premature senescence results in an average reduction in life expectancy of several days. This is potentially a very important phenomenon in female vector mosquitoes, as it is only the small proportion of very old individuals which can transmit malaria, due to the length of time taken during the parasite extrinsic development cycle. Daily adult survival is a crucial component of any measurement of vectorial capacity, and even modest reductions in this figure can result in a dramatic decrease in the likelihood of malaria transmission. The use of insecticides in vector control programs has always been a key method of reducing daily adult survival, but this is currently only assessed on the immediate death rate. In areas where resistance

has emerged, the expected effect of insecticide use on vectorial capacity is assumed to be much reduced if immediate mortality alone is considered. Findings from this study suggest that the use of pyrethroid insecticides might be expected to maintain a significant effect on malaria transmission due to premature senescence, even if immediate mortality is reduced by resistance in the vector population. This could have important policy implications in vector control and would favour continued use of pyrethroids, in the absence of suitable alternatives, even after resistance has developed.

Significant effects of sublethal exposure on fecundity per unit time were not observed in this study. The average number of eggs produced per gonotrophic cycle per surviving female was no different between control and treatment groups with any of the compounds used. Likewise, there was no variation in egg viability and larval hatch rate identified. However, the reduced longevity in females exposed to pyrethroids did result in a significantly reduced total mean lifetime egg production per female, due to the fact that these individuals completed fewer gonotrophic cycles. In terms of disease transmission, this effect is considered unlikely to have any significance, as the majority of females in a population are unlikely to complete all gonotrophic cycles theoretically possible in their lifetime due to the low daily life expectancy.

Leg fracture is reported as a common occurrence in individuals exposed to many pyrethroids. However, it seems to have no detrimental effect on egg production. This may be due to the fact that all these experiments were conducted on anopheline species, where the hind legs have no role in oviposition, in contrast to *Culex* species, where the hind legs are used in egg raft formation.

15.2. Effects on activity and behaviour

Experimental recording of female flight activity in relation to contact to, and post-exposure of, pyrethroid insecticides both confirmed earlier reports of immediate irritancy, and led to new evidence of post-exposure lethargy. It was no surprise that an immediate increase in flight activity was observed when females were placed on surfaces treated with these insecticides. It was interesting to see that in the case of permethrin, the activity was spontaneous, whereas in the case of lambda-cyhalothrin and deltamethrin, abnormal activity began only after a period of several minutes had elapsed. This is in itself important, as a high irritancy may result in an insect not resting on a treated surface long enough to pick up a lethal dose, thus reducing efficacy. This presumably explains the fact that the latter compounds have been found to be more suited to residual spraying than permethrin.

Probably the most important finding concerning activity from this study is that females previously exposed to sublethal doses of pyrethroids show significantly reduced evening host seeking the day after treatment. This observation, made initially in the actograph, was subsequently reinforced in tests of free-flying mosquitoes where host seeking behaviour of treated females was found to be substantially inhibited. In terms of disease transmission, this factor is likely to be of significance, as females contacting a pyrethroid treated surface such as an impregnated bed net, may not only be unsuccessful in feeding on that occasion, but may be less likely to seek a host later that night or the following evening. Reducing potential host feeds and effectively increasing time between feeds are both likely to reduce transmission. The fact that host seeking flight activity reduction is only transient: strong at 24 hours, moderate at 48 hours and not significant at 72 hours, makes it likely that this is due to residual effects of insecticides on the flight muscles or, perhaps more likely, the nervous control of these muscles.

Observation of both increased and abnormal probing response in females shortly after contact with pyrethroids is not dissimilar to that reported in tsetse flies (Chadd & Brady, 1982). It is a well documented phenomenon that exposure to this class of compounds induces hyper-excitation, twitching and convulsions as a consequence of repetitive firing of neurons in the peripheral nervous system. The increased activity of such a basic response is probably attributable to this direct effect on nervous control. The impact of this phenomenon on disease transmission will probably vary depending upon which disease is involved. As there is reportedly a limited number of sporozoites available for injection during a single bite (Wright, 1969; Sterling *et al.*, 1973), because these are limited by physical dimensions of the salivary ducts, it is unlikely that increased probing activity caused by pyrethroid exposure will affect malaria transmission. However, in the case of some arboviral diseases, there is evidence that increased probing or duration of feeding can increase inoculation of the virus, and thus potentiate transmission (Grimstad *et al.*, 1980). This may need to be considered where these compounds are used as skin applied repellents or in vector control of arbovirus vectors. It is interesting that this initial increase in probing activity subsides rapidly and is followed by a marked reduction in response just a few hours post-contact. In terms of disease transmission, this too is likely to prove beneficial, as a female having contacted a treated surface on entry to a house, such as an eaves curtain or wall, is less likely to probe and feed later that evening. On a large scale, one might expect that this could lead to an overall reduction in the human sporozoite inoculation rate.

15.3. Effects on vector / parasite interaction

The effects which have been found of pyrethroids on the development of the malaria parasite within the vector mosquito could have an important effect on disease control. The preliminary studies on rodent malaria provided evidence that sublethal exposure to all 3 of the compounds tested had a detrimental effect on parasite development. This effect was

observed when contact with insecticide occurred shortly before, during or shortly after an infective feed. Even during the initial experiments it was clear that timing of exposure and infection had an important influence on outcome. In experiments where pyrethroid exposure occurred before the infective feed, there was a smaller reduction in numbers of individuals becoming infected, although the decreased oocyst burden was much more significant. Those individuals receiving a sublethal exposure shortly after taking an infective feed were found to have a much more pronounced reduction in overall numbers becoming infected, compared to controls. When a more realistic experimental exposure procedure was used, i.e. taking a feed on an infective mouse through pyrethroid impregnated netting, a significant reduction in infection levels was still seen. Exactly how this may relate to human malaria transmission was then emphasised using similar experimental techniques with cultured *Plasmodium falciparum*, again with significant results. With pyrethroids in widespread use around the world for malaria vector control programmes one might expect this phenomenon to play a role in control of disease transmission, however, it would be difficult to quantify the effect in the field. However, one could imagine a situation where there was a greater reduction in malaria than anticipated or expected, based on standard measures of impact on the vector. Furthermore, entomological surveys might observe a reduction in oocyst / sporozoite infection rates in a vector population beyond that which can be explained by changes in numbers or age composition. Whilst both of the above may be attributable to the sublethal effects of pyrethroids on vector infectivity, they could equally be due to other factors, such as personal protection of infected / susceptible hosts, or the selective kill of older females by cumulative pesticide exposure. Under such circumstances, it is difficult to see how these effects could be disentangled and measured in the field.

Having established that there is an effect of sublethal exposure to pyrethroids on host / parasite interactions, an attempt was made to elucidate the mechanism involved.

The experiments which did most to help identify this process involved the delay of exposure over pre-set time periods post infective feed. The findings were very clear cut: if an infective feed occurred before 18 hours post exposure there was a strong reduction in subsequent infection rates. If the feed took place 24 hours after exposure there was a smaller effect, and if it was delayed until 48 hours, no effect was seen. This indicates that the critical process affected by the insecticide exposure occurs between 18 and 48 hours after the start of parasite development within the vector. During this period there are a number of important biological events taking place, e.g. development of the ookinete following exflagellation, penetration of the peritrophic membrane and ookinete migration through the midgut wall. Each of these are complex processes and are under regulation by a number of biochemical factors. Several different potential targets were evaluated. Direct effects on exflagellation was ruled out, as was the inhibition of trypsin, which may have accounted for reduced chitinase activity leading to poor intracellular penetration by the ookinete. Although studies utilising radiolabeled permethrin showed that the compound was present in the mosquito gut after blood feeds, no direct anti-malarial activity was found in culture assays which might have explained the phenomenon. Some research groups have been developing techniques to culture ookinetes in the laboratory (Targett, 2001, personal communication). Perhaps the best course of action to uncover the mode of action of pyrethroids on parasite development in the vector may be to utilise this advance. In particular, observing the effect of these substances on enzymatic and biochemical processes which initiate and facilitate ookinete penetration of the mid gut wall and subsequent development into oocysts may prove fruitful.

It is particularly interesting that whilst sublethal effects on longevity, fecundity, activity and behaviour have been reported in many insect species using a wide range of insecticide classes, it is only the pyrethroids which seem to have this effect of inhibition of parasite development in the vector.

15.4. Conclusion

Evidence presented in this thesis suggests that there are likely to be additional benefits, over and above the anticipated acute mortality of vectors, when malaria control programmes utilise pyrethroid insecticides. In particular, the three key phenomena of premature senescence, host seeking inhibition and indirect effects on parasite development, are likely to afford a degree of protection even in situations where immediate mortality is limited by resistance. Attempts should be made to identify and monitor such effects in future control programmes using these compounds.

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